ENDOCRINOLOGY

VOLUME 41

JULY-DECEMBER, 1947

EDITORIAL BOARD

E. B. ASTWOOD C. H. BEST J. S. L. BROWNE C. G. HARTMAN

F. L. HISAW E. C. KENDALL F. C. KOCH

C. N. H. LONG H. B. VAN DYKE

E. W. DEMPSEY, *Managing Editor*

25 Shattuck St., Boston, Mass.

PUBLISHED FOR

THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

UNIVERSITY OF MICHIGAN LIBRARIES

Copyright, 1947, by the Association for the
Study of Internal Secretions, Inc. All rights reserved.

George Banta Publishing Company
Menasha, Wisconsin

CONTENTS OF VOLUME 41

NO. 1, JULY, 1947

LESLIE, STEPHEN H., AND ELAINE P. RALLI
The Effect, in Rats, of High Fat Diets on the Renal Excretion of Water and Antidiuretic Substances 1

HUFFMAN, MAX N., AND ARTHUR GROLLMAN
The Relative Estrogenic Activity of Compounds Related to Estriol 12

HEMPHILL, R. E., AND M. REISS
Regulation of Endogenous Cortin Production 17

KIMELDORF, DONALD J., AND A. L. SODERWALL
Changes Induced in the Adrenal Cortical Zones by Ovarian Hormones ... 21

KATZMAN, PHILIP A., N. J. WADE AND EDWARD A. DOISY
Gonadotropic Modifying Action of Sera of Animals Treated with Hypophyseal Extracts 27

MEYER, ROLAND K., S. W. SOUKUP, W. H. MCSHAN AND CLYDE BIDDULPH
Succinic Dehydrogenase in Rat Ovarian Tissues during Pregnancy and Lactation 35

STAFFORD, ROBERT O., W. H. MCSHAN AND ROLAND K. MEYER
Acid and Alkaline Phosphatases in Ovarian Tissues of the Rat during Pregnancy and Lactation 45

PABST, MARVIN L., RUTH SHEPPARD AND MARVIN KUIZENGA
Comparison of Liver-Glycogen Deposition and Work Performance Tests for the Bio-Assay of Adrenal Cortex Hormones 55

STANLEY, MALCOLM M., AND E. B. ASTWOOD
Determination of the Relative Activities of Antithyroid Compounds in Man Using Radioactive Iodine 66

ELFTMAN, HERBERT
Response of the Alkaline Phosphatase of the Adrenal Cortex of the Mouse to Androgen 85

PFEIFFER, CARROLL A.
Gonadotrophic Effects of Exogenous Sex Hormones on the Testes of Sparrows 92

NOTES AND COMMENTS

ANDERSON, EVELYN, ERNEST W. PAGE AND CHOH HAO LI
The Development of a Refractory State to the Adrenocorticotrophic Hormone 105

CANZANELLI, ATTILIO, RUTH GUILD AND DAVID RAPPORT
The Influence of the Thyroid on Alanine Synthesis by the Liver 108

NO. 2, AUGUST, 1947

BUCHWALD, KENNETH W., AND LEONA HUDSON
The Biochemical Effects of Injections of Sex Hormones into Hypophysectomized Rats 111

COLE, H. H., AND R. B. CASADY
Studies on Indices and Causes of Prolificacy in Rats 119

TRENTIN, J. J., AND C. W. TURNER
Effect of Adrenalectomy on the Mammary Gland of the Castrated and Estrogen Treated Castrated Male Rat 127

LUDEWIG, STEPHAN, AND ALFRED CHANUTIN
The Adrenal Cholesterol and Ascorbic Acid Contents after Injury 135

BAKER, BURTON L., AND NEWTON B. EVERETT
The Effect of Diethylstilbestrol on the Anterior Hypophysis of Thyroidectomized Rats 144

HOOKER, CHARLES W., AND THOMAS R. FORBES
A Bio-Assay for Minute Amounts of Progesterone 158

INGLE, DWIGHT J., MILDRED C. PRESTRUD, CHOH HAO LI AND HERBERT M. EVANS
The Relationship of Diet to the Effect of Adrenocorticotrophic Hormone upon Urinary Nitrogen, Glucose and Electrolytes 170

MADDOCK, WILLIAM O., AND CARL G. HELLER
Gonadotrophic Hormone: Protection Against Inactivation 177

TEPPERMAN, JAY, AND HELEN M. TEPPERMAN
Effect of Gonadotrophins on the Cholesterol Content of the Testes of Immature Rats 187

UNIVERSITY OF MICHIGAN LIBRARIES

NOTES AND COMMENTS
KRICHESKY, BORIS, AND S. J. GLASS
Vaginal Cornification in Spayed Rats by Intravaginal Application of Non-Estrogenic Substances 196
JAILER, JOSEPH W.
A Fluorometric Method for the Determination of Estrogens 198
BRÜCKMANN, G.
The Diabetogenic Activity of Alloxan in Old and Young Rats 201
HECHTER, OSCAR, AND ZAREH HADIDIAN
Hyaluronidase Activity of Spermatozoa 204
ASSOCIATION NOTICE 206

NO. 3, SEPTEMBER, 1947

INGLE, DWIGHT J., JAMES E. NEZAMIS AND MILDRED C. PRESTRUD
The Effect of Diethylstilbestrol upon Alloxan Diabetes in the Male Rat 207
HARTMAN, F. A., K. A. BROWNELL AND R. A. KNOUFF
Increased Fat Factor Production after Adrenal Enucleation 213
DVOSKIN, SAMUEL
Intracellular Colloid Droplets as a Basis for Thyrotrophic Hormone Assay in the Chick 220
WHITE, ABRAHAM, AND THOMAS F. DOUGHERTY
Role of the Adrenal Cortex and the Thyroid in the Mobilization of Nitrogen from the Tissues in Fasting 230
DEANE, HELEN WENDLER, AND ROY O. GREEP
A Cytochemical Study of the Adrenal Cortex in Hypo- and Hyperthyroidism 243
BERMAN, DOREEN, MARJORIE SYLVESTER, ELEANOR C. HAY AND HANS SELYE
The Adrenal and Early Hepatic Regeneration 258
NOTES AND COMMENTS
CLEGHORN, ROBERT A.
Species Difference in the Cortical Hormone Requirement of Adrenalectomized Dogs and Cats 265
FLEISCHMANN, WALTER, AND I. ALFRED BRECKLER
Mitotic and Wound Healing Activities of the Corneal Epithelium in Thiouracil Treated and Thyroidectomized Rats 266
SZEGO, CLARA M., AND SIDNEY ROBERTS
The Failure of Splenectomy to Influence the Uterine Response to Alpha-Estradiol in Castrate and Partially-Hepatectomized Castrate Rats 269
ASSOCIATION NOTICE 272

NO. 4, OCTOBER, 1947

PECKHAM, B. M., AND R. R. GREENE
Attempts to Produce Deciduomata in the Pregnant Rat 273
PECKHAM, B. M., AND R. R. GREENE
Production of Secondary Deciduomata in the Castrate and Lactating Rat .. 277
BIRD, S., L. I. PUGSLEY AND M. O. KLOTZ
The Quantitative Recovery of Synthetic Estrogens from Tissues of Birds (Gallus Domesticus), the Response of the Birds' Testis, Comb and Epidermis to Estrogen and of Humans to Ingestion of Tissues from Treated Birds 282
WOLFF. J., AND I. L. CHAIKOFF
The Relation of Thyroxine to Total Iodine in the Thyroid Gland 295
BARSANTINI, J. C., AND GEORGES M. C. MASSON
Effects of Steroids on Lactation 299
MURPHREE, R. L., E. J. WARWICK, L. E. CASIDA AND W. H. MCSHAN
Influence of Reproductive Stage Upon the Fertility of Gonadotrophin-Treated Female Rabbits 308
BOYARSKY, LILA H., HARRIET BAYLIES, L. E. CASIDA AND R. K. MEYER
Influence of Progesterone Upon the Fertility of Gonadotrophin-Treated Female Rabbits 312
NOTES AND COMMENTS
SZEGO, CLARA M., AND SIDNEY ROBERTS
The Determination of Protein-Bound Blood Estrogen 322
SULLIVAN, CLEORA DONOVAN, AND JAMES WILLIAM ARCHDEACON
The Effects of a Large Dose of Para-Amino-Benzoic Acid on the Body and Endocrine Gland Weights of Rats: Toxic Manifestations 325

CLARK, GEORGE
Threshold Bleeding and the Sex Skin in the Castrate Female Chimpanzee 327
FORBES, THOMAS R.
Testis Transplantations Performed by John Hunter 329
DVOSKIN, SAMUEL
Further Observations on the Thyroxine-Like Action of Elemental Iodine in the Rat 331
Announcement of the 1948 Meeting of the Association for the Study of Internal Secretions 334

NO. 5, NOVEMBER, 1947

VALLE, J. R., S. B. HENRIQUES AND OLGA B. HENRIQUES
The Assay of Androsterone by the Chick Comb Weight Method and the Renal Excretion of Androgens by the Cebus Monkey 335
CRAFTS, ROGER C., AND BURNHAM S. WALKER
The Effects of Hypophysectomy on Serum and Storage Iron in Adult Female Rats 340
CHRISTENSEN, BØRGE GOTFRED, AND CLAUS MUNK PLUM
The Significance of the Endocrine Glands in the Ripening of Reticulocytes .. 347
TEPPERMAN, JAY, HELEN M. TEPPERMAN, BARBARA W. PATTON AND LESLIE F. NIMS
Effects of Low Barometric Pressure on the Chemical Composition of the Adrenal Glands and Blood of Rats 356
EVERETT, JOHN W.
Hormonal Factors Responsible for Deposition of Cholesterol in the Corpus Luteum of the Rat 364
MCKAY, DONALD G., AND DAVID ROBINSON
Observations on the Fluorescence, Birefringence and Histochemistry of the Human Ovary During the Menstrual Cycle 378
SAWYER, C. H., J. E. MARKEE AND W. HENRY HOLLINSHEAD
Inhibition of Ovulation in the Rabbit by the Adrenergic-blocking Agent Dibenamine 395
DVOSKIN, SAMUEL
The Spontaneous Formation of Intracellular Colloid Droplets in Surviving Chick Thyroid Tissue 403
WILSON, HILDEGARD, AND PRISCILLA CARTER
Stabilization of the Alcoholic Potassium Hydroxide in Colorimetric 17-Ketosteroid Determinations 417
BERN, HOWARD, AND MICHAEL KLEIN
The Urethral Smear as an Indicator of Sex Hormone in Male Rabbits 422
FOGLIA, V. G., N. SCHUSTER AND R. R. RODRÍGUEZ
Sex and Diabetes 428
NOTES AND COMMENTS
R-CANDELA, JOSE LUIS AND L. G. URGOITI
Complement in Alloxan Diabetes 435

NO. 6, DECEMBER, 1947

REISS, M., AND D. S. REES
Pituitary and Carbohydrate Metabolism of the Brain 437
MCQUEENEY, A. J., L. L. ASHBURN, F. S. DAFT AND R. FAULKNER
Tissue Changes and Sodium Balance in Pantothenic Acid-Deficient Rats .. 441
DORFMAN, RALPH I.
Studies on the Bioassay of Hormones. The Relative Potencies of Testosterone Propionate and Testosterone-17-β-Diethyl Amino Ethyl Carbonate Hydrochloride 451
DORFMAN, RALPH I., AND BETTY L. RUBIN
Studies on the Bioassay of Hormones. The Assay of Chorionic Gonadotrophin from Human Pregnancy Urine and Serum 456
DORFMAN, RALPH I., ALBERT M. POTTS AND MARY L. FEIL
Studies on the Bioassay of Hormones: The Use of Radiosodium for the Detection of Small Quantities of Desoxycorticosterone 464
DORFMAN, RALPH I., BENJAMIN N. HORWITT, REGINALD A. SHIPLEY, WILLIAM R. FISH AND WILLIAM E. ABBOTT
Metabolism of the Steroid Hormones: Studies on 17-Ketosteroids and Androgens 470

UNIVERSITY OF MICHIGAN LIBRARIES

LANDAUER, WALTER
Potentiating Effects of Adrenal Cortical Extract on Insulin-Induced Abnormalities of Chick Development 489
LIPSETT, MORTIMER B., AND RICHARD J. WINZLER
Effects of Vitamin A Deficiency on Thyroid Function Studied with Radioactive Iodine 494
BRADBURY, JAMES T.
Ovarian Influence on the Response of the Anterior Pituitary to Estrogens .. 501
NOTES AND COMMENTS
SULMAN, F., AND M. PEREK
Influence of Thiouracil on the Basal Metabolic Rate and on Molting in Hens 514
GORDAN, GILBERT S., AND HENRY W. ELLIOTT
The Action of Diethylstilbestrol and Some Steroids on the Respiration of Rat Brain Homogenates 517
Index 519

ENDOCRINOLOGY

VOLUME 41 JULY, 1947 NUMBER 1

THE EFFECT, IN RATS, OF HIGH FAT DIETS ON THE RENAL EXCRETION OF WATER AND ANTIDIURETIC SUBSTANCES

STEPHEN H. LESLIE AND ELAINE P. RALLI

From the Department of Medicine

NEW YORK UNIVERSITY COLLEGE OF MEDICINE

IT HAS BEEN repeatedly observed that a high fat, low protein diet will cause fatty infiltration of the liver in rats and, if continued for a long enough period of time, will result in a disease of the liver consisting of portal cirrhosis with occasional necrosis (Gyorgy 1944, Webster 1941, Gyorgy and Goldblatt 1941). Shay, Kolm, and Fels (1945) reported that when rats fed this diet were subjected to water tolerance tests, they excreted less of the administered water than rats fed a normal diet. These investigators did the tolerance tests after the animals had been on the diets for periods up to eighteen days. The physiological state of the kidney was measured by the excretion of phenol red in the urine after it had been injected intraperitoneally. The diet did not impair the ability of the kidneys to excrete phenol red, although a definite depression in water excretion was observed very early in the course of the diet. The authors suggested that impaired liver function might be the mechanism responsible for the altered renal excretion in the animals. Clinically severe liver disease is known to be associated with a decreased urine volume (Gilbert and Lerebouillet 1901; Pick 1929–30) and Adlersberg and Fox (1943) have reported the use of a water tolerance test as one measure of liver function in both dogs and patients. As part of our studies on the disturbance of water balance occurring in cirrhosis of the liver, we have subjected rats, maintained for prolonged periods on the high fat-low protein diet, to water tolerance tests at intervals during the dietary period. Certain modifications of the diet were introduced in or-

Received for Publication April 21, 1947.

This Research was aided by grants from The Nutrition Foundation and The Lederle Laboratories. . . .

Copyright, 1947, by the Association for the Study of Internal Secretions

UNIVERSITY OF MICHIGAN LIBRARIES

der to simulate the therapeutic measures employed in the treatment of patients with severe liver disease.

PROCEDURE

A total of 180 rats were used in the studies. They were bred in the laboratory and were fed the Nu-Chow diet until placed on the experimental diet. At 75 to 100 days of age the high fat diet was begun. This consisted of 38% fat (primex), 8% casein, 47% cane sugar, 2% cod liver oil, and 5% of a mineral mixture which contained no NaCl. A supplement of the vitamin B complex was added to the feeding cup in each cage of four rats daily. The rats were divided into four groups: Group A received the diet without NaCl, Group B received the diet and a solution of 1% NaCl in the drinking water, Group C received the diet without NaCl but were given intraperitoneally three times weekly 1 cc. of a diluted crude liver extract (Lederle). The extract was diluted (1 cc. of liver extract to 32 cc. of distilled water) so that the amount injected was equivalent to the amounts administered to patients with cirrhosis of the liver. Group D received the diet plus the 1% NaCl solution and, in addition, the diluted liver extract. The undiluted liver extract contained per cc. 14.8 mgm. total nitrogen, 208 mgm. total solids, and 0.05 mgm. histamine diphosphate. At intervals during the course of the diet water tolerance tests were done on rats in each of the groups. The procedure was as follows: the evening before the tests, the rats were deprived of food and water. The morning of the test the rats were hydrated by stomach tube with a volume of water equal to 5 per cent of their body weight. Twelve rats from each group were tested and were placed, in groups of four, in metabolism cages. The urine was collected in small graduated cylinders and the volumes were recorded at 15-minute intervals, beginning 30 minutes after hydration was started. The experiments were continued until 50% of the ingested water had been excreted or, up to a total of 300 minutes, for as the diet period progressed, the time required for 50% excretion was so great, in some groups, that it was felt that other factors might be influencing urine output. The chloride concentration was determined in the urine samples representing 25% of the ingested water. When this excretion was not reached, the determination was done on the entire sample collected. Phenol red tests were done intraperitoneally by injecting 1 mgm. of the phenol red in 2 cc. of N/saline rendered alkaline with 0.1 N NaOH. The water tolerance tests were done after the rats had been on the diet for 7, 35–36, 53–55, 91–95, and 138 days. The phenol red tests were done after 44, 67, and 109 days of the diet.

RESULTS

The results of the water tolerance tests, in all groups, are shown graphically at each period of the diet (figs.1–5). When normal rats,

75 to 100 days of age, are hydrated to 5 per cent of their body weight, the ingested water is excreted rapidly and 50% of the water is excreted after 135 minutes (Fig. 1). The excretory curve is steep and uniform. In all of the four experimental groups, the nature of the curve was affected after 7 days of the diet. At this time the excretion was least affected in the rats in Group B (diet plus saline), and the excretion curve was most significantly affected in Group A (who received the diet alone). Except for the rats in Group B, the excretory curve after 135 minutes tended to level off so that the time required for 50% excretion was definitely prolonged. In Groups A and D the

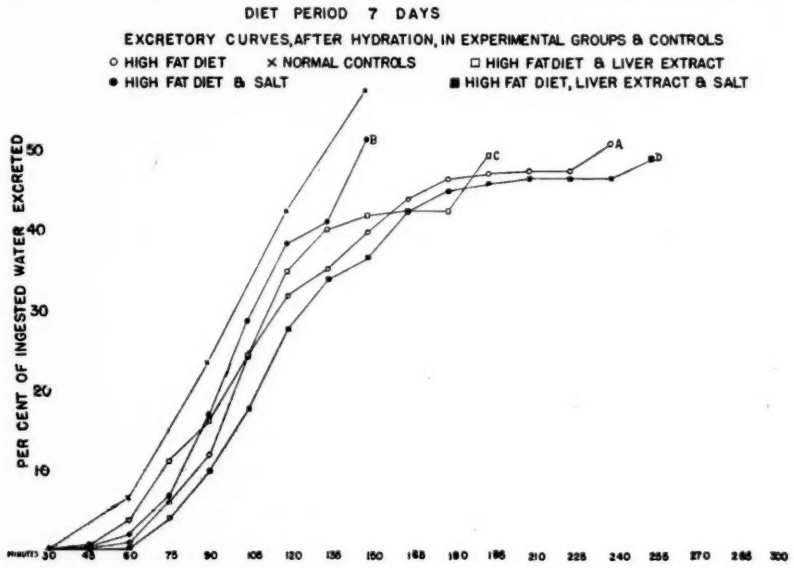

DIET PERIOD 7 DAYS
EXCRETORY CURVES, AFTER HYDRATION, IN EXPERIMENTAL GROUPS & CONTROLS
○ HIGH FAT DIET
× NORMAL CONTROLS
□ HIGH FAT DIET & LIVER EXTRACT
● HIGH FAT DIET & SALT
■ HIGH FAT DIET, LIVER EXTRACT & SALT
PER CENT OF INGESTED WATER EXCRETED
50
40
30
20
10
A
B
C
D
MINUTES 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300

FIG. 1.

plateau persisted for 45 minutes. Tests done after the period of the diet had been continued for 36 days showed a greatly delayed urine output from the onset and the steep gradient in the excretory curve was no longer present. The greatest change was observed in the rats not receiving NaCl, regardless of whether they were given the liver extract or not (Groups A and C). In both of these groups the excretory curves, after an initial rise, remained flat and 50% excretion was never reached. Apparently the NaCl intake had a direct bearing on the excretion of urine for, as is shown in Groups B and D, the excretory curve although significantly altered, was not as greatly depressed as in the groups not receiving any NaCl. Liver extract had no effect on the rate of excretion of ingested water in the group not receiving salt (Group C).

UNIVERSITY OF MICHIGAN LIBRARIES

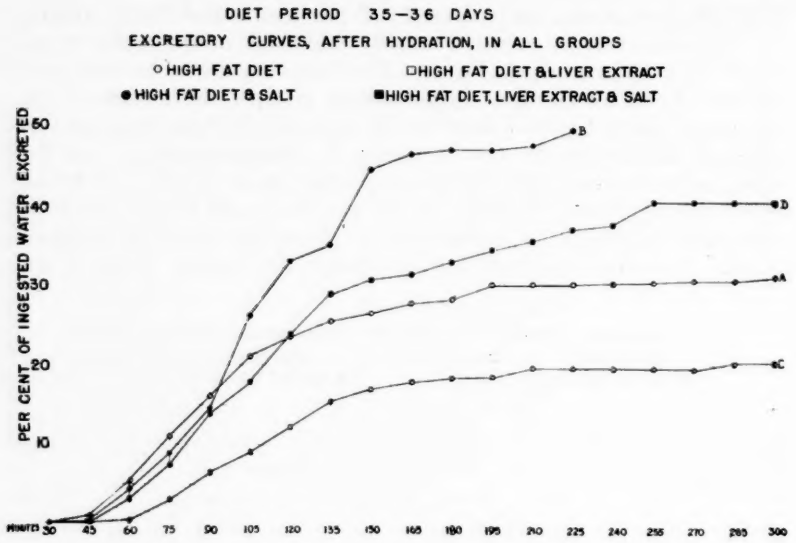

DIET PERIOD 35–36 DAYS
EXCRETORY CURVES, AFTER HYDRATION, IN ALL GROUPS
○ HIGH FAT DIET
□ HIGH FAT DIET & LIVER EXTRACT
● HIGH FAT DIET & SALT
■ HIGH FAT DIET, LIVER EXTRACT & SALT
PER CENT OF INGESTED WATER EXCRETED
MINUTES
B
D
A
C

FIG. 2.

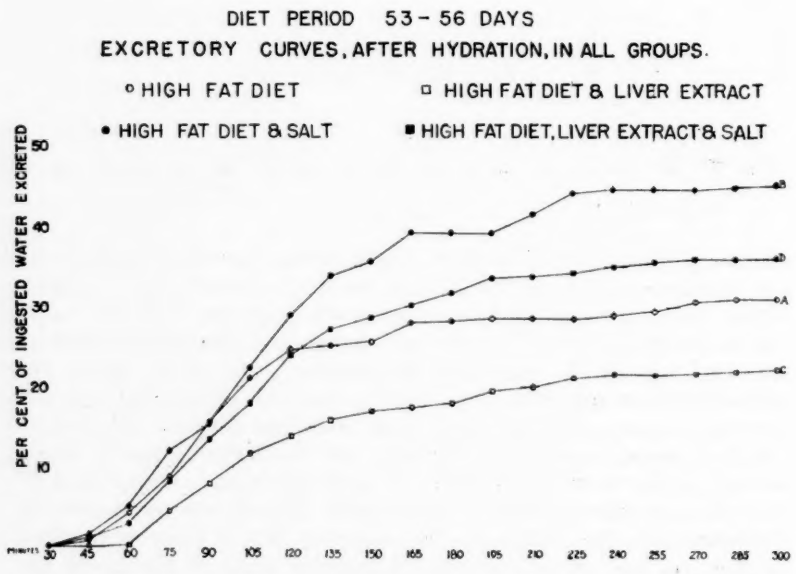

DIET PERIOD 53 - 56 DAYS
EXCRETORY CURVES, AFTER HYDRATION, IN ALL GROUPS.
○ HIGH FAT DIET
□ HIGH FAT DIET & LIVER EXTRACT
● HIGH FAT DIET & SALT
■ HIGH FAT DIET, LIVER EXTRACT & SALT
PER CENT OF INGESTED WATER EXCRETED
MINUTES
B
D
A
C

FIG. 3.

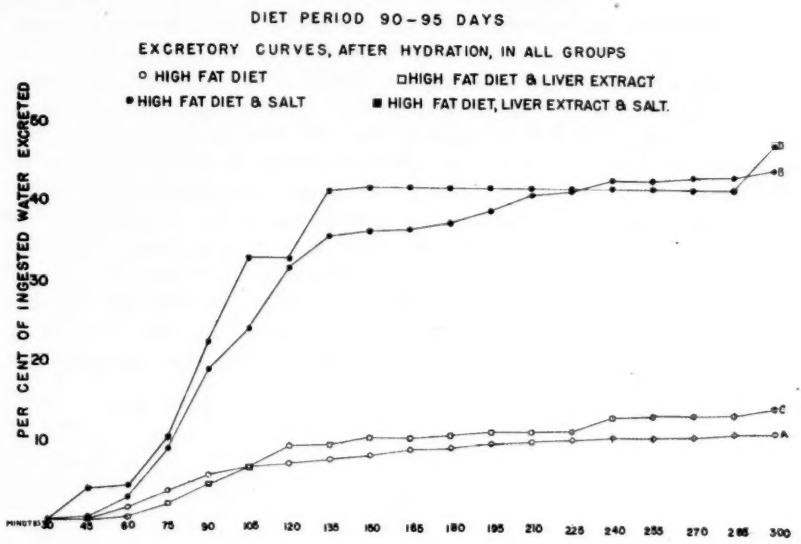


FIG. 4.

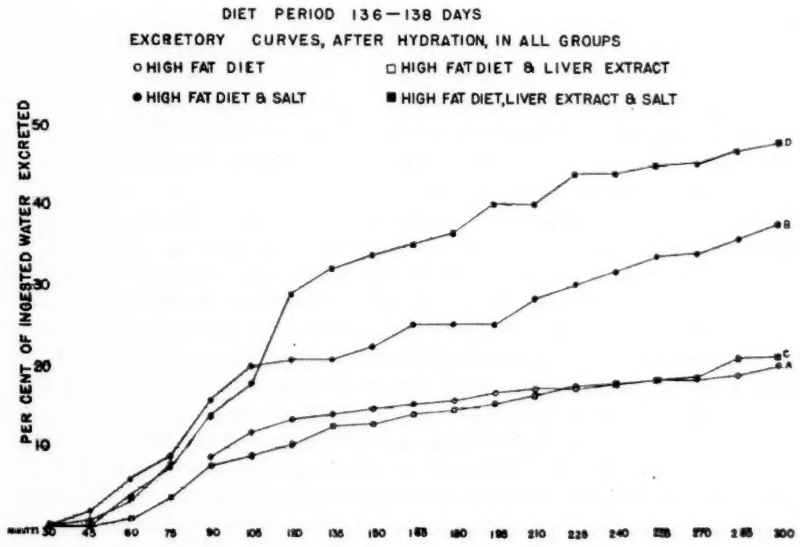


FIG. 5.

UNIVERSITY OF MICHIGAN LIBRARIES

As the diet period continued the excretory curve, in the rats not receiving salt, continued to be depressed and the initial rise seen within the first 90 minutes in the earlier period of the diet also flattened out. After 90 days of the diet, only 20% of the ingested water was excreted within the 300 minutes of the test. In Groups B and D, receiving salt, the excretory curve did not change to the extent that it did in the Groups A and C. There was, however, a distinct flattening out of the curve but there was a greater excretion of urine in the early period of the test so that the plateau was on a higher level. In Group D, receiving both salt and injections of liver extract, the rate of urine excretion was not further depressed after the 56th day of the diet. In fact, the rate of urine flow was improved at the 90th and 138th days of the test. This was the only evidence that the injections of

TABLE I. CHLORIDE EXCRETION DURING WATER TOLERANCE TESTS[1]

Period of diet	A High fat			B High fat salt			C High fat "liver"			D High fat, "liver," salt		
	Av. wt. gms.	Vol. urine cc.	Micro eq. Cl per 100 gms.	Av. wt. gms.	Vol. urine cc.	Micro eq. Cl per 100 gms.	Av. wt. gms.	Vol. urine cc.	Micro eq. Cl per 100 gms.	Av. wt. gms.	Vol. urine cc.	Micro eq. Cl per 100 gms.
7	169	8.3	9.0	163	9.0	11.3	167	9.0	24.6	169	10.2	20.3
35–36	157	8.2	11.7	162	8.5	10.4	155	6.5	9.6*	171	9.1	16.1
53–56	145	7.6	5.8	159	9.3	10.5	147	6.4	3.7*	166	9.1	3.2
91–95	146	3.1	4.2*	147	8.8	8.2	142	4.4	9.8*	163	8.2	9.1
138	134	5.5	6.2*	132	6.8	11.6	138	5.4	6.3*	159	9.7	9.9

[1] Chloride determinations were done on the urine sample representing 25% of the ingested water except in the starred figures (*) where, owing to the depressed urine output, the chloride excretion was determined on the total volume of urine excreted within the 300 minutes of the collection period.

liver extract might have some effect but obviously it was also associated with the administration of NaCl. The importance of NaCl is illustrated by Group C, (receiving the extract but without salt) in which group the urine excretion was markedly depressed.

It is clear that the high fat diet is associated with a retardation of the excretion of water output, and that the degree of this retardation is influenced by the intake of NaCl.

In view of the differences in the excretory curve during the water tolerance tests, the excretion of chloride is interesting. The results for all the groups are given in Table I and are reported in microequivalents per 100 gm. of rat weight. The urine samples analyzed were those representing 25 per cent excretion of the ingested water, as this represented a constant comparable amount and when 25 per cent excretion was not reached, the determinations were done on the entire volume excreted within the period of the test. The results are averages of the experiments done at each period of the diet. The fact that all of the rats were deprived of water and food for a period of 17 hours before the tests undoubtedly operated to equalize the chloride excretion. The rats in Groups A and B excreted less chloride after 7 days on the diet than did the rats in Groups C and D. At the 35 day test, the greatest excretion of chloride was in the rats in Group C. After this time the chloride excretion in Groups A and C, not receiving

salt, remained low while in the groups on salt the excretion ranged from 8.2 to 11.6 microequivalents per 100 gm. of rat weight. The differences in chloride excretion are partly due to the differences in the urine output in the groups and, in comparison, the chloride excretion was less affected by the diet than was the urine excretory curve.

The excretion of phenol red following intraperitoneal injections is reported in Table II. The excretion was measured after 90 minutes and after 180 minutes. The results are reported as the per cent of the amount injected that was excreted. The normal rats excreted an average of 46% in the first 90 minutes, 10.5% in the second 90 minutes, and a total 57% for the entire period. After 44 days of the diet, the

TABLE II. PHENOL RED EXCRETION

Diet	Duration of diet in days											
	Controls % PSP excreted Minutes			44 days % PSP excreted Minutes			67 days % PSP excreted Minutes			109 days % PSP excreted Minutes		
	0–90	90–180	Total	0–90	90–180	Total	0–90	90–180	Total	0–90	90–180	Total
Controls	50.3 42.5	7.5 13.5	57.8 56.0									
High Fat				57.0	3.2	60.2	5.3	55.3	60.6	29.8	4.1	33.9
High Fat Salt				62.0	5.4	67.4	3.7	Lost		30.5	4.1	34.6
High Fat-Liver Extract				36.2	14.0	50.2	1.5	74.3	75.8	32.7	4.0	36.7
High Fat-Liver & Salt				49.2	9.6	58.8	53.5	4.4	57.9	39.0	7.5	46.5

excretion in Groups A and B (receiving no liver extract) within the first 90 minutes was increased to 57 and 62 per cent respectively. The total excretion for the 180 minutes was slightly greater than in the normal rats, averaging 60 and 67 per cent. In Groups C and D, at this time, the total excretion was 50 and 58 per cent. After 67 days of the diet the per cent of the phenol red excreted for the total period of time was normal or somewhat greater than normal in all the groups but in Groups A, B, and C only a small fraction of the dye was excreted during the first 90 minutes. We have no explanation for this. In Group D, 53.4 per cent of the dye was excreted in the first 90 minutes. After 109 days of the diet there was a significant decrease in the total per cent of the dye excreted in all groups and the greatest part of this was excreted within the first 90 minutes. The rats in Group D showed the least change excreting 39 per cent in 90 minutes and a total of 46.5 per cent for the entire period of 180 minutes. Shay et al. (1945) did the phenol red tests after their rats had been on the diets for 105 days and collected the urine for 180 minutes. They report from 55 to 62 per cent of the dye excreted at that time. The experimental diet that they used contained a salt mixture plus Vegex, which is an extract of Brewer's Yeast and contains salt. Possibly our Group D, receiving both 1% NaCl and liver extract, most nearly approaches the experimental conditions of their animals. The decreased excretion of the dye in Groups A, B, and C seems to us to indicate some degree of kidney damage, which is known to occur when this type of diet is continued for very long periods. Rats sacrificed after 140 days of the diet showed some degree of kidney

UNIVERSITY OF MICHIGAN LIBRARIES

damage but the most outstanding finding was the pathology of the liver which consisted of both fatty infiltration and fibrosis.

In view of our observation that an antidiuretic substance could be extracted from the urines of patients with cirrhosis of the liver and ascites, (Ralli et al. 1945) we collected urine from rats on the high fat diet and assayed it for its antidiuretic titre. The urines of rats from Groups A and B were selected for assay. At the time the urine was collected the rats had been on the diet for more than 150 days. Postmortem examinations were done on the animals to ascertain the state of the livers and kidneys. For the period of urine collection, the rats were placed in individual metabolism cages. It was necessary to collect the urine for more than 24 hours as rats on the high fat diet without salt excrete very small amounts of urine. As controls, urines from normal rats on the Nu Chow diet were assayed. Some of the control rats were allowed unlimited amounts of water and the other controls were allowed only 5 cc. of water per 24 hours for a period of several days, so that the urine volumes would be similar to those of the rats on the experimental diets. A total of three days urine was used for assay. In order to avoid fecal contamination the urine was collected in fractions daily and kept in the icebox until ready for assay. Extraction was done as previously described (Ralli, Robson, Clarke, and Hoagland 1945) and the antidiuretic assay was done by the method of Burn (1931), 1 cc. of the extracted urine being injected intraperitoneally into normal rats hydrated to 5 per cent of their body weight. The results are given in Figure 6. The excretion is charted for each 30 minute interval in order to show the nature of the excretory curve following the injection of the various urine extracts. The extract from the urine of normal rats had no antidiuretic effect and the excretory curve is very steep and uniform. The extract from the urines of normal rats on a very limited water intake had a definite antidiuretic effect. The line drawn through the centre of the points shows a delayed excretion of urine, and urine flow did not begin to increase steadily until after 150 minutes. The extract from the urines of the rats on the high fat diet, regardless of whether they received 1% NaCl or not, had a very marked antidiuretic effect. In fact, the extracts from the urines of some of the high fat rats almost completely suppressed urine flow for the period of 300 minutes of observation. The chloride excretion was determined on the urines that were excreted during the assay period, representing 25 per cent of the ingested water. The extract from the urine of the normal rats, which had no antidiuretic effect, resulted in a chloride excretion of 13.8 microequivalents per 100 gm. of rat weight. The extract from the urines of the dehydrated normal rats resulted in a chloride excretion of 11.2 microequivalents per 100 gm. of rat weight, and the extract from the urines of the rats fed the high fat diet resulted in a chloride excretion of 7.8 microequivalents per 100 gm.of rat weight. This latter is in the range of the chloride excretion obtained in the urine samples of the

rats on the high fat diets during the water tolerance tests (Table I).

The livers of the rats were examined at intervals during the diet period. Fatty infiltration was the predominant finding until the diet period was well advanced. After 130 days of the diet, fibrosis was pres-

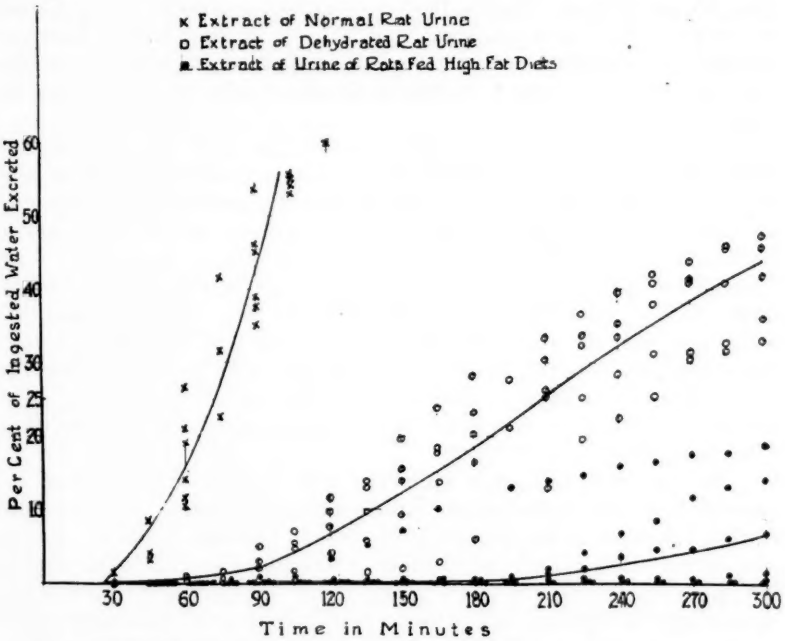


FIG. 6. The Effect of the Injection of the Antidiuretic Substances Extracted from the Urines of (a) Normal Rats, (b) Dehydrated Rats and (c) Rats on the High Fat Diets, on Urine Output in Hydrated Normal Rats.

ent in varying degrees but this was still accompanied by varying amounts of fatty infiltration. A complete analysis of the pathological data will be the subject of a later report.

DISCUSSION

The diets used in these studies produced the changes in the livers of the rats that have been described by other observers (Gyorgy, 1944; Webster, 1941). As the period of the diet progressed, there was a progressive depression in the urine excretory curve following water tolerance tests. This change was most marked in the rats deprived of salt. The intraperitoneal injection of a crude liver extract had no influence on the water tolerance tests in the rats that did not receive salt. After 135 days on the diet the water tolerance tests were less retarded in Group D, receiving both salt and the injections of liver extract, than in any of the other groups. This was a consistent finding.

Antidiuresis is an expression of many physiological factors and it may well be that in rats on this type of diet the associated malnutrition is a contributing factor. The rats in all groups lost weight during the period of the diet. The greatest average loss of weight was in Group A (40 gms.). The average weight loss in Group B and C was less, 33 and 31 gms. The smallest average loss of weight was in Group C, 13 gms. It is, however, significant that, in spite of similar losses of weight, the excretion of water was less disturbed in Group B, receiving salt, than in Group C receiving the diet and liver extract but no salt.

The antidiuretic titre of the urine of the rats on the diet was high, which is consistent with the state of antidiuresis. Gilman and Goodman (1937) have demonstrated that the urine of rats subjected to dehydration, contained appreciable amounts of an antidiuretic substance. Our findings on dehydrated rats are in agreement with theirs and the finding of an even greater antidiuretic effect with the urine extract from the rats on the high fat diet, suggests that this substance may be active in suppressing urine flow in these animals. Investigators working on the identity of the A.D. substance in urine have suggested that it is derived from the posterior pituitary as it cannot be found in the urine of hypophysectomized rats or dogs (Pickford 1945, Verney 1946).

The changes in chloride excretion were influenced by the low intake of NaCl and reflected the decreased urine volume that occurred during the tests in the experimental rats in Groups A and C. As the diet period continued, the chloride excretion also fell in Group D which suggests that the high fat diet may be associated with some disturbance in sodium and chloride metabolism as well as with water excretion. As the period of the diet progressed some of the low chloride excretion may have been due to impairment of kidney function, but this would not explain the low chloride values of the urines occurring at tests done on the 56th day of the diet. The phenol red excretion was the least affected in Group D but after 138 days of the diet, the rats in all groups showed changes in this test.

Delayed diuresis is not uncommon in either clinical or experimental liver damage and the water tolerance tests on patients with severe hepatitis reported by Adlersberg and Fox (1943), have some of the same characteristics observed in the rats on the high fat diets. In both instances there was delayed diuresis and the total amount of urine excreted represented only a fraction of the water administered.

In view of the influence of the administration of NaCl on the character of the excretory curve in the rats and on the per cent of the ingested water excreted, one might speculate that withholding salt from patients with cirrhosis of the liver and ascites may further enhance the delayed diuresis observed in such cases. The high fat diet used in the production of fatty infiltration of the liver experimentally is almost the antithesis of the diet used in the treatment of cirrhosis

of the liver. In the latter condition a highly nutritious diet, rich in protein and carbohydrate and slightly restricted in fat, is the choice whereas the experimental high fat-low protein diet is deficient in amino acids and so it would be unlikely that a crude liver extract would offset the effect of the deficiency. It is interesting, in view of the conditions of the experiment, that the addition of salt to the diet and the combined effect of salt and liver extract modified the nature of the excretory curve.

Our observations support the suggestion of other observers (Pick 1929, Adlesberg and Fox 1943, Shay et al. 1945) that liver damage is associated with a disturbance in water balance as reflected by the rate of urine excretion and that this occurs before any pathological changes are present in the kidney.

SUMMARY

Repeated water tolerance tests were done on rats fed high fat-low protein diets. The rats were divided into four groups according to the supplement added to the diet. Group A received the diet alone; Group B, the diet plus a solution of 1% NaCl; Group C, the diet plus injections of liver extract; and Group D, the diet plus 1% NaCl and injections of liver extract. The rate of urine excretion was altered in all four groups after the rats were placed on the high fat diet. The changes consisted in a delay in the onset of diuresis and a 'leveling off' of the excretory curve, when the latter was plotted as the per cent of the ingested water excreted at given intervals of time following hydration. The most striking depression in urine flow occurred in the rats in Groups A and C, in which NaCl was *not* given. The excretory curve was the least affected in the rats receiving the 1% NaCl solution *plus* injections of liver extract.

The urines of the rats on the high fat diet, either with or without NaCl, when extracted and injected into hydrated normal rats, were found to exert a marked antidiuretic effect. This is consistent with the occurrence of delayed diuresis observed in the experimental rats and substantiates the observation of other observers that an increase in the A.D. substance is present in the urine when a state of antidiuresis exists.

REFERENCES

ADLERSBERG, D., AND C. L. FOX, JR.: *Ann. Int. Med.* 19: 642. 1943.
BURN, J. H.: *Quart. J. Pharm.* 4: 517. 1931.
GILBERT, A., AND P. LEREBOULLET: *Compt. rend. Soc. de Biol.*, iii: 229. 1901.
GILMAN, A., AND L. GOODMAN: *J. Physiol.* 90: 113. 1937.
GYORGY, P.: *Amer. J. Clin. Path.* 14: 67. 1944.
GYORGY, P., AND H. GOLDBLATT: *Proc. Soc. Exper. Biol. and Med.* 46: 492. 1941.
PICK, E. P.: *The Regulation of Water Metabolism.* Harvey Lectures, New York 30: 32. 1929.
PICKFORD, MARY: *Phys. Rev.* 25: 573. 1945.
RALLI, E. P., J. S. ROBSON, D. CLARKE AND C. L. HOAGLAND: *J. Clin. Invest.* 24: 316. 1945.
SHAY, H., R. KOLM AND S. S. FELS: *Gastroenterology* 4: 257. 1945.
VERNEY, E. B.: *Lancet* 251: 739. 1946.
WEBSTER, G.: *J. Clin. Invest.* 20: 440. 1941.

THE RELATIVE ESTROGENIC ACTIVITY OF COMPOUNDS RELATED TO ESTRIOL[1,2]

MAX N. HUFFMAN AND ARTHUR GROLLMAN

From the Departments of Biochemistry, Pharmacology, and Experimental Medicine, The Southwestern Medical College

DALLAS, TEXAS

THE EXISTENCE of a reversible equilibrium between estrone and α-estradiol and the conversion of the former to the latter in the human being is well established. The pathway of the conversion of estrone to estriol is still, however, a matter of speculation. A knowledge of the exact course of estrogen metabolism is important for it may lead to the production of more active naturally occurring estrogens as well as to better concepts of the possible role of these compounds in carcinogenesis and in the body economy in general (Grollman, 1947). We have determined the estrogenic activities of various compounds, prepared by partial synthesis from estrone, which may conceivably fall along the normal or abnormal course of estrone metabolism. The results permit certain speculations regarding the pathway for the transformation of estrone to estriol.

MATERIALS AND METHODS

The following compounds have been used in the present study: estradiol, estriol, estrone, "Ruzicka's triol," 16-keto-α-estradiol, isoestriol-A (Huffman and Darby, 1944), and 16-ketoestrone.[3] The estradiol, estriol, and estrone were known pure preparations which were available in our laboratory. By "Ruzicka's triol" we have reference to the compound designated as $\Delta^{1,3,5}$-estratriene-triol-(3, 16α, 17α) by Ruzicka, Prelog, and Wieland (1945). A generous supply of this compound was supplied us by Dr. Prelog for whose cooperation we are indebted. The remaining three compounds, 16-keto-α-estradiol, isoestriol-A, and 16-ketoestrone, were prepared in this laboratory. All the compounds tested were dissolved in 95 per cent aqueous ethyl alcohol and diluted with normal saline to give the required concentrations for assay.

Inasmuch as the results of estrogenic assays are contingent on the methods utilized, we have adopted two standard procedures for these studies (Curtis, *et al.*, 1944; Pugsley and Morrell, 1943). The compounds were tested for their capacity to induce (1) premature opening of the vagina in immature rats and (2) an estrous smear in the adult castrated rat. In each assay a comparison was made of the activity of the unknown with estradiol. The amount of the unknown material necessary to induce a positive reaction in 50 per

Received for publication April 25, 1947

[1] Presented before the Society for the Study of Internal Secretions at its annual meeting, Atlantic City, June 6, 1947.

[2] Aided by grants from the U. S. Standard Products Company, Woodworth, Wisconsin.

[3] The synthesis of 16-ketoestrone, soon to be published, follows closely the method described for 16-ketoestrone-3-methyl ether (Huffman, 1947).

cent of the experimental animals was compared with the amount of estradiol required to induce a similar response. In this way, any variability in the animals used for a given assay was corrected for by the result obtained with the estradiol accepted as a standard of potency.

The rats used were of the piebald Evans-McCollum strain reared in our laboratory. Twenty animals were used in each assay for the unknown and an equal number for the standard. Injections in the immature animals were made subcutaneously twice daily for three days; in the adult castrated animals, twice at 7-hour intervals.

RESULTS

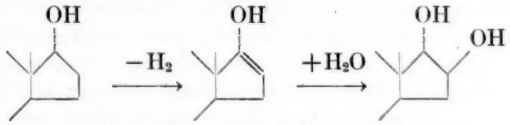
The relative activities of the seven compounds used in the present study are given in Table 1. The values for estradiol, estriol, and estrone are in good agreement with those reported by previous workers. Prelog, Ruzicka, and Wieland (1945) reported the activity of their compound (designated in the table as "Ruzicka's triol") as approximately equal to that of naturally occurring estriol. Our own studies confirm this for the immature rat. However, as assayed on the adult mature rat this compound is only approximately one-half as active as estriol as shown in Table 1.

TABLE 1. RELATIVE ESTROGENIC ACTIVITY OF CERTAIN STEROIDS. VALUES IN THE TABLE ARE THE AMOUNTS NECESSARY TO INDUCE A POSITIVE REACTION IN 50 PER CENT OF THE ANIMALS

	Activity	
	As tested on immature rats	As tested on castrated adult rats
	μg.	μg.
Estradiol	0.1	0.03
Estriol	0.3	0.3
Estrone	1.0	0.24
"Ruzicka's triol"	0.32	0.6
16-keto-α-estradiol	1.5	0.7
Isoestriol-A	1.5	0.5
16-ketoestrone	2.1	1.1

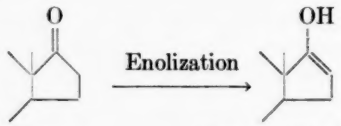
DISCUSSION

The two currently held views regarding the probable pathway of transformation of estrone to estriol in the organism are those advanced by Marrian (1938–9) and by Fieser (1936). According to Marrian, α-estradiol is dehydrogenated to the enolic form of estrone which in turn is hydrated to form estriol as indicated in the following formulae in which only steroid ring D is shown:



α-estradiol Enolic form of estrone Estriol

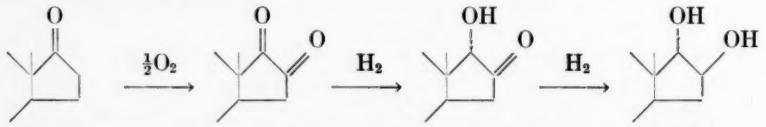
Fieser, likewise, has postulated that estriol is formed in the organism by hydration of an enolic form of estrone as shown in the above formulae. He assumed that the enol form of estrone is formed directly by enolization of estrone.



Estrone Estrone (Enol Form)

It has, however, so far been impossible to prepare by any known chemical method a 17-enol derivative of a 17-ketosteroid. Apparently enolization of the 17-ketosteroid takes place with considerable difficulty. This failure to enolize is probably due to the strain that an olefin linkage would impose upon steroid ring D, the C/D ring bridge very likely being *transoid* in nature.

Marrian has also suggested that the compound known as 16-ketoestrone might be the end product of simultaneous estrogen and progestin metabolism and that this diketone might be further oxidized in the body to products at present unknown. One might consider 16-ketoestrone itself as a precursor of estriol if one assumes that it is first reduced to give 16-keto-α-estradiol by the addition of one molecule of hydrogen and that the resulting 16-keto-α-estradiol is then further reduced by the addition of another molecule of hydrogen to give estriol. 16-ketoestrone has been prepared and its estrogenic activity determined as shown in Table 1. It is a labile substance being both oxidized and reduced easily. 16-keto-α-estradiol might also be thought of as being formed directly from α-estradiol by oxidation, that is, by the insertion of an oxygen atom at C_{16}, although this pathway seems much less likely since in estrone itself the steroid position 16 is a much more reactive one. We may formulate the above transformations as follows:

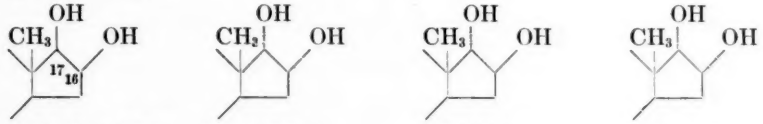


Estrone 16-ketoestrone 16-keto-α-estradiol Estriol

It is to be noted that in both the immature and the adult castrated rat the order of estrogenic activity is: 16-ketoestrone <16-keto-α-estradiol <estriol. 16-keto-α-estradiol is also, surprisingly enough, less active than estrone in both types of assay. 16-keto-α-estradiol may be identical with the ketonic steroid which Pearlman and Pincus (1943) found in urine and which these observers showed contains an $-$OH group other than the usual phenolic one of estrone. Smith and Smith

(1941) have also presented evidence for the existence of a new compound, of possible importance in the catabolism of estrogen, which on reduction gives rise to a more active substance (16-keto-α-estradiol to estriol?). The administration of 16-ketoestrone or 16-keto-α-estradiol to an experimental animal followed by an examination of the estrogen fraction of the excreta would aid in establishing or disproving this newly suggested origin of estriol in the organism.

By the reduction of 16-ketoestrone four possible stereoisomers of estriol, in regard to the configurations at C_{16} and C_{17}, are possible. These four isomers are given in the following diagrams in which only ring D is shown:



"Ruzicka's triol" Isoestriol-A Estriol Unknown

Evidence for the 16α,17α-configuration for isoestriol-A and for the 16β,17α-configuration for estriol will be presented in detail elsewhere (Huffman, unpublished). Prelog, Ruzicka, and Wieland believed that their compound (which we have designated as "Ruzicka's triol") had the 16α,17α-structure, but this is unlikely. The compound is apparently the 16β,17β-derivative as shown above. The fourth member of the series, which so far has not been synthesized, is the second of the two possible *trans* forms. These isomeric compounds are of theoretical interest in connection with the view that an abnormal metabolism of estrone is a causative factor in cancer of the genital organs.

A comparison of the relative activities of estriol, "Ruzicka's triol", and isoestriol-A shows that they are in the approximate ratio of 1 to 0.9 to 0.2, respectively, as determined in the immature rat and in a ratio of 1 to 0.5 to 0.6, in the adult. The *trans*-configuration of the glycol is thus associated with the maximum activity, and hence it would be of great interest to examine the remaining unknown *trans* member of the four epimers.

From the reduction of 16-keto-α-estradiol two epimers are possible,—estriol and isoestriol-A. It is interesting to note that in the immature animal estriol is five times as active as isoestriol-A whereas in the mature animal it is less than twice as active.

SUMMARY

The relative estrogenic activities of the following compounds were determined by assay on the immature and the castrated adult rat: estradiol, estriol, estrone, "Ruzicka's triol", 16-keto-α-estradiol, isoestriol-A, and 16-ketoestrone. Estriol is the most active of the three known α-glycols obtained by reduction of 16-ketoestrone. Es-

triol is also more active than either 16-keto-estrone or 16-keto-α-estradiol.

The probable pathway of the conversion of estrone to estriol in the body is discussed. It is suggested that 16-ketoestrone is formed from estrone by oxidation and that the dione is reduced through 16-keto-α-estradiol to estriol.

REFERENCES

CURTIS, J. M., E. WITT AND L. K. KNUDSEN: *Endocrinology* 34: 149, 1944.

FIESER, L. F.: Chemistry of the Natural Products Related to Phenanthrene, Reinhold Publ. Corp., New York, 1936, pp. 187–255.

GROLLMAN, A.: Essentials of Endocrinology, J. B. Lippincott, Philadelphia, 2nd edition, 1947.

HUFFMAN, M. N., AND H. H. DARBY: *J. Am. Chem. Soc.* 66: 150. 1944.

HUFFMAN, M. N.: *J. Biol. Chem.* 167: 273. 1947.

MARRIAN, G. F.: Harvey Lectures, Williams and Wilkins Co., Baltimore, 1938–9, pp. 37–54.

PEARLMAN, W. H., AND G. PINCUS: Vitamins and Hormones, Academic Press, New York, 1943.

PRELOG, V., L. RUZICKA AND P. WIELAND: *Helv. Chim. Acta* 28: 255. 1945.

PUGSLEY, L. I., AND C. A. MORRELL: *Endocrinology* 33: 48. 1943.

SMITH, O. W., AND G. V. S. SMITH: *Endocrinology* 28: 740. 1941.

REGULATION OF ENDOGENOUS CORTIN PRODUCTION

R. E. HEMPHILL AND M. REISS
Biochemical & Endocrinological Research Department of Bristol Mental Hospitals

INVESTIGATION in the output of cortin should furnish important information about adrenal cortex activity in normal and pathological states.

Ever since the existence of adrenal cortex hormones was known, responsibility for numerous regulatory processes was attributed to the adrenal cortex, although changes in the content of adrenal cortex compounds in body fluids could not be easily demonstrated. Several reliable biological methods for determining cortin in body fluids, particularly urine, were worked out, but all required large numbers of experimental animals. The margin of error of these methods is considerable, and their results prove too circumstantial for investigations in series.

A method for direct chemical determination of cortical compounds is therefore especially valuable. By making use of the reducing properties of side chains of all these substances, chemical determination of them in purified extracts of the body fluids is possible. Talbot, Saltzman, Wixom, and Wolfe (1945), and Heard, Sobel, and Venning (1946), have thus estimated excretion of cortin compounds in the urine; the correlation between the chemical and biological assays is satisfactory.

We have been interested in determination of cortical substances in blood, adrenals, and other organs, after certain procedures.

METHODS

Blood of groups of 25–50 rats was collected over oxalate after decapitation. Blood of patients was taken from the antecubital vein and oxalated. Thirty to fifty cc. of blood proved sufficient for a single determination. The blood was slightly acidified (pH 5.5) and precipitated with four times its volume of redistilled absolute alcohol. The solution was filtered on a Buchner funnel, and the precipitate twice more extracted. The extracts were evaporated under vacuum at 50° C. The residue was re-extracted with alcohol-ether mixture (3:1), the solution filtered and evaporated. The residues were then extracted exhaustively with chloroform four times, and the extract collected in a separating funnel. From here onwards, the purification was continued in accordance with the method described by Talbot et al. (1945). The chloroform extracts were washed three times with one-tenth of their

Received for publication April 29, 1947.

volume of chilled n/10 NaOH, and three times with distilled water, each wash being re-extracted with equal volumes of chloroform which was added to the original chloroform solution before carrying out the next wash. The chloroform solution was now evaporated under vacuum at 50° C. The residues were taken up in about 3 ml. benzene (thiophen-free and redistilled), and transferred to separating funnels, this process being repeated with 3 similar volumes of benzene. The combined benzene extracts were extracted 8 times with distilled water, the benzene being discarded afterwards. The water extracts were extracted 3 times with 10 ml. chloroform for each extraction. The combined chloroform extracts were dried over sodium sulphate and then evaporated in vacuo at 50° C.; any remaining traces of chloroform were removed by adding about 2 ml. of methyl alcohol (purified after semicarbazide treatment) and evaporated to dryness. The residues were each taken up in 2–4 ml. methyl alcohol. Duplicate samples of 0.5 ml. of the methyl-alcohol extracts were taken and the reducing power of the extracts estimated by the Hagedorn-Jensen blood-sugar method. We prefer the use of this method to the colorimetric methods used by Talbot et al. (1945) and Heard et al. (1946), which, in our hands, showed certain drawbacks for cortin determination (turbidity and unspecific reactions). Measured by the Hagedorn-Jensen method, 1 mgm. of desoxycorticosterone acetate was equivalent to a reducing power of 0.4 mgms. glucose. The amounts of reducing substances in the extracts are, therefore, expressed in milligrams D.O.C.A.

The amount of corticosteroid in the adrenals was estimated by a similar method. All the adrenals of each group were kept on ice during the preparation, then weighed together and afterwards ground with sand and chloroform. The chloroform extract was poured off, and the adrenal tissue re-extracted in the same manner 3 times with further amounts of chloroform.

RESULTS

Table I shows the results of experiments in rats, investigating the influence of corticotrophic hormone on cortin content of blood and adrenals. Fifty male and 50 female rats were each injected with 8 units of corticotrophic hormone 3 times daily for one day; and a second series of 50 males and 50 females with 4 units 3 times daily for two days. They were killed and the blood collected over oxalate. A similar number of untreated controls for each series of 50 were killed and investigated at the same time.

The values recorded show that the cortin content of the blood rose slightly after 24 hours, equally in the males and females. After 48 hours, the cortin content increased further, the treated animals having 100% more cortin in blood than the animals of the control group. The cortin content of the adrenals was increased after 24 hours, but decreased by over 70% after 48 hours.

Table II shows the changes in blood cortin content of two male patients treated with corticotrophic hormone. A rise up to 800%, 24 and 48 hours after injection of hormone, was recorded.

Table III shows the influence of exposure to cold on the cortin content of blood. Groups of 24 female or male animals were kept for

TABLE I. CORTIN CONTENT OF ADRENALS AND BLOOD OF MALE AND FEMALE RATS WEIGHING 160–180 G., AFTER TREATMENT WITH CORTICOTROPHIC HORMONE

No. and sex of rats	Average body weight in g.	Treatment		Total weight adrenals in mg.	Total volume blood in ml.	mg. "Cortin"	
		Units corticotrophic hormone injected three times daily	Duration of treatment before dissection	Used for analysis		in 100 g. adrenals	in 100 ml. blood
50 ♀	171	Controls	24	2610	165	17	0.303
50 ♀	180	8	24	2678	165	26	0.369
50 ♀	175	Controls	48	2419	161	19	0.284
50 ♀	179	4	48	2962	160	4	0.885
50 ♂	170	Controls	24	1558	190	11	0.385
50 ♂	183	8	24	1646	210	23	0.442
50 ♂	167	Controls	48	1524	160	12	0.350
50 ♂	175	4	48	1690	195	3	0.610

TABLE II. BLOOD CORTIN CONTENT OF PATIENTS

	Day	Treatment	"Cortin" mg in 100 ml. blood
Patient T.	1	—	0.21
	2	120 units Cortrophin intramuscularly	
	3	120 units Cortrophin intramuscularly	1.07
	4	—	1.61
Patient B.	1	—	0.435
	2	60 units Cortrophin intramuscularly	
	3	60 units Cortrophin intramuscularly	0.91
	4	—	1.03

TABLE III. CORTIN CONTENT OF ADRENALS AND BLOOD OF MALE AND FEMALE RATS WEIGHING 90–100 G. KEPT FOR DIFFERENT PERIODS OF TIME IN THE COOLROOM (5–6° C.)

No. and sex of rats	Number of hours the rats were kept in the coolroom at 5–6° C.	Total weight adrenals in mg.	Total volume blood in ml.	mg. "Cortin"	
		Used for analysis		in 100 g. adrenals	in 100 ml. blood
24 ♀	0 (Controls)	780	64	26	0.218
25 ♀	19	883	73	29	0.465
25 ♀	42	897	75	27	0.480
25 ♀	70	918	80	76	0.854
25 ♂	0 (Controls)	649	76	9	0.420
18 ♂	19	475	53	25	0.944
14 ♂	42	395	42	16	0.462
16 ♂	70	455	46	57	1.445

different lengths of time in a cool-room at 5–6° C. The cortin content of the blood started to rise after 19 hours and after 70 hours was increased by over 300%. There was little increase in the cortin content of the adrenals of the females during the first 42 hours, but a considerable increase after 70. In the males there was a slight increase in the first 42 hours, but an increase of over 500% after 70 hours.

DISCUSSION

Since only certain morphogenetic actions of the corticotrophic hormone were known, and there was no definite proof of the corticotrophic action, namely mobilisation of cortin, estimation of blood cortin after treatment with corticotrophic hormone was regarded as important.

Depletion of cortin in the adrenals after treatment with corticotrophic hormone provides a paralled to the depletion of iodine in the thyroid after treatment with thyrotrophic hormone. It appears that in both cases the rate of mobilisation of hormone is greater than the rate of new production. It remains to be seen whether there are more than one corticotrophic hormone fractions responsible for production and mobilisation of the peripheral hormones. It may be the case that such corticotrophic hormone fractions were produced by the pituitary during the cold experiments, for here, by contrast with what obtained after treatment with a purified corticotrophic hormone fraction, the cortin content of the adrenals increased to a greater extent, although there was a considerable rise in blood cortin in both cases.

It is interesting to note that, while the average adrenal weight and cortin content of the female control groups listed in Tables I and III are higher than the average adrenal weight and cortin content of the male control group, the blood cortin content is lower.

SUMMARY

A method for the determination of cortin-like steroids with reducing side chains in the blood has been described. The blood content of these reducing cortin-like steroids was increased in two series of 50 male and 50 female rats respectively, and in two male patients, after treatment with corticotrophic hormone. The cortin contents of the adrenals of the rats decreased 48 hours after the commencement of treatment.

The content of cortin in blood and adrenals of rats kept in the cold increased considerably.

REFERENCES

Heard R. D. H., Sobel H. and Venning E. G.: *J. Biol. Chem.* 165: 699. 1946.

Talbot N. B., Saltzman A. H., Wixom R. L. and Wolfe J. K.: *J. Biol. Chem.* 160: 535. 1945.

CHANGES INDUCED IN THE ADRENAL CORTICAL ZONES BY OVARIAN HORMONES

DONALD J. KIMELDORF AND A. L. SODERWALL
From the Department of Biology, University of Oregon
EUGENE, OREGON

THAT THE FEMALE gonadic hormones influence the adrenal glands in rodents has been demonstrated for the female rat (Ellison and Birch, 1936; Anderson, 1934), mouse (Danner, 1938) and guinea pig (Allen and Bern, 1942). Changes in the adrenal cortex have also been noted during the reproductive cycle indicating a correlation between ovarian physiology and adrenal cortical functions (Anderson and Kennedy, 1932; Zalesky, 1934; Zuckerman, Bourne and Lewes, 1938). In all of these studies the initial effect of estrogen injection was a marked hypertrophy of the adrenal cortex. Simular treatment using progesterone yielded conflicting pictures with Clausen (1940) reporting an actual decrease in cortex while Selye, Brown and Collip (1936) and Selye (1940) noted no effect.

Accepting the hypothesis presented by Bennett (1939) and Deane and Greep (1946) indicating specialized zones for active production of adrenocortical hormones, it is the purpose of this report to present data concerning the influence exerted by natural ovarian hormones on the individual cortical layers of the adrenal.

The adrenal gland in the guinea pig presents suitable material for both macroscopic and histological examinations. The three cortical layers are particularly well-defined and have proven very sensitive to hormonal influences. The relatively large size of the gland lends an added advantage.

MATERIALS AND METHODS

Thirty-five virgin female guinea pigs of age 4½ to 5½ months and weight 450–650 grams were observed for normal cyclic estrous behavior. Twenty-three of this group were spayed and allowed a recuperative period of 21 days. Each was then tested for normal estrous response to administration of ovarian hormones[1] utilizing the standardized technique of Boling, Young and Dempsey (1938). All animals exhibited capacity to respond normally to female sex hormones.

The spayed animals were placed in the following groups: Group I included nine animals which received estrogen injections. Three spayed ani-

Received for publication May 1, 1947.

[1] Estrogen, in the form of Progynon-B, and progesterone, in the form of Proluton, were supplied by the Schering Corporation, Inc., Bloomfield, N. J., through the courtesy of Dr. Erwin Schwenk.

mals received three subcutaneous injections of five R. U. estrogen at 48 hour intervals. Forty-eight hours after the last injection they were sacrificed, the adrenals removed, sectioned and stained for histological examination. Three spayed animals of the same age and weight class as above received ten R. U. estrogen at 48 hour intervals. This dosage has previously been found adequate to sensitize the guinea pig prior to injection of progesterone for inducing the behavioral heat reaction (Collins, Boling, Dempsey and Young, 1938). The glands were removed and prepared for study. Three spayed animals of the same class received twenty R. U. estrogen at 48 hour intervals.

Group II included nine progesterone-injected spayed females: Three animals received three subcutaneous injections of 0.05 mg. at 48 hour intervals. Three animals received 0.1 mg. at similar intervals. This dosage is sufficient to induce heat in an estrogen-conditioned animal (Collins, Boling, Dempsey and Young, 1938). Three animals received 0.2 mg. progesterone.

Group III consisted of 12 normal animals in the 450–650 gram weight class. This constituted the normal control group. All animals in this group were known to be in the diestrous condition when sacrificed.

Group IV was composed of five animals standardized similarly to group III but were spayed and sacrificed in the same manner as the experimental groups. Groups III and IV received injections of sesame oil instead of the hormones administered to the experimental groups.

All glands were fixed in Bensley's bichromate-sublimate-formalin mixture for 24 hours and hardened in 3% bichromate for three weeks. The left adrenal was used in each case as a further standardization. Sections were made at four to eight micra depending upon the subsequent histological or cytological studies. Two routine stains were employed: Regaud's iron-hematoxylin or Krichesky-Mallory triple stain. For some of the glands, frozen sections were stained with Scharlach-R for examination of lipoid inclusions within the cortex.

Each gland was weighed to the nearest tenth-milligram. These weights were compared with the body weights and a table prepared showing the relative milligram weight of adrenal to gram weight of body for a description of effects. Histological examination included the observation and measurements of the three cortical layers. Sections with largest cross-sectional area were projected on a screen and outline tracings made of the cortical zones. These tracings were measured by a planimeter set at a constant arm length and are summarized in Tables 1 and 2. These measurements were reproducible within an error of one per cent.

In the evaluation of results, Fisher's test for significant differences between small groups was applied. When the t-value of the two groups being compared was less than 2.2, the difference was not considered statistically significant.

RESULTS

Table 1 shows the average relative weight of adrenals as influenced by the treatment indicated. (Relative adrenal weight refers to milligram of adrenal per gram body weight. For simplification of presentation this figure was multiplied by a constant, 10^7.) Each figure represents the average value for the particular group. The control group was significantly the lowest with an average relative weight of

2345. A comparison of the estrogen-injected group with the controls shows clearly the marked effect exercised by the female hormone on the relative adrenal weight. This increase in relative weight is correlated with a substantial increase in cortical width as shown by the column "Entire Cortex".

The relative adrenal weight of the progesterone-treated animals,

TABLE 1. SUMMARY OF GROUP AVERAGES FOR RELATIVE ADRENAL WEIGHTS, CORTEX SIZE AND MEDULLA SIZE. THE "t" VALUES* REFER TO COMPARISONS WITH THE NORMAL CONTROLS. CORTEX AND MEDULLA MEASUREMENTS ARE IN PLANIMETER UNITS

Condition of animals	No.	Adr. wt. / Body wt. $\times 10^7$	"t" value	Cortex size	"t" value	Medulla size
Controls	12	2345	—	16,949	—	558
Castrates	5	2558	1.6	19,364	0.5	528
Estrogen treated	9	3554	59.6	25,710	23.6	483
Progesterone treated	9	2874	46.4	20,427	10.3	519

$$* t = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{n_1 Sx^2 + n_2 Sy^2}{n_1 + n_2 - 2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}.$$

TABLE 2. SUMMARY OF GROUP AVERAGES FOR THE SIZE OF THE INDIVIDUAL CORTICAL ZONES. THE "t" VALUE HAS THE SAME REFERENT AS IN TABLE 1

Condition of animals	No.	Glom. zone	"t" value	Fasc. zone	"t" value	Ret. zone	"t" value
Controls	12	622	—	6331	—	2924	—
Castrates	5	746	1.8	6001	1.1	3265	1.3
Estrogen treated	9	718	8.1	4236	92.8	5542	57.8
Progesterone treated	9	824	74.8	6283	1.7	3319	16.5

like those receiving estrogen, was significantly greater than the normal controls and castrates. As indicated in Table 1 this effect in the cortical width was considerably less than the estrogen-treated group.

In confirmation of other workers (Allen and Bern, 1942) it is to be noted that the planimeter determinations indicate no significant effect on the medulla following castration or sex hormone administration. The castrate group showed no significant difference in relative adrenal weight and cortex size when compared with the normal controls.

Table 2 summarizes planimeter measurements relative to the individual cortical layers in each experimental and control group. The results of the measurements give a picture of a significantly increased width of the zona glomerulosa following injection of the hormones. Of the hormones administered, progesterone exerted a much greater influence on the glomerulosa area than did estrogen. It is of interest to note that the respective dosages usually employed to condition and induce heat in the guinea pig were found to produce

the greatest effect on the glomerulosa. The actual significance of this observation awaits further confirmation.

The effects produced by the individual hormones on the fascicular zone revealed a very marked decrease in area of the estrogen-treated females when compared to the controls. No such effect was observed in the progesterone-treated animals. With reference to the zona reticularis the estrogen effect is very pronounced as an increased zonal area in comparison to the controls. The same zone was significantly enlarged in the progesterone-treated animals. No apparent change in size of the individual zones was noted after castration in the guinea pig.

The cellular detail of each zone was compared among the three groups. In the estrogen series the fascicular cells became hypertrophied. The microliposomes became more consistently of a large size. Even more pronounced is the frequency of large macroliposomes. In those animals which received 20 R. U. of estrogen, the fascicular layer appeared to be fenestrated with macroliposomes. The progesterone series resembled more closely the controls in the number of macroliposomes but approached the estrogen series in cell size. Frozen sections stained with Scharlach-R revealed an increased amount of lipoid inclusions in the hypertrophied cells.

The reticularis zone of the controls consisted largely of small irregular, deeply staining cells with an occasional light staining vacuolated cell. These dark shrunken cells had all the characteristics of degeneration and pycnosis. It was these dark staining cells which increased tremendously in number to produce the enlarged reticular zone of the estrogen-injected animals. The progesterone-injected animals showed little increase in the frequency of these cells.

The glomerulosa zone consisted of spherical clusters of cells. Each cell contained very finely dispersed lipoid vacuoles. These cells were hypertrophied particularly in the progesterone series.

Within each experimental group there was a shift in the widths of cortical zones which is worthy of note. In response to the estrogen treatment, a dramatic alteration in zonal areas was noted. The fasciculars zone became greatly reduced and was accompanied by a comparably strong shift towards a widened reticularis zone. In response to progesterone, there was a pronounced enlargement in the glomerulosa with a smaller increment in the reticularis zone. No significant change was noted in the fascicular zone size.

DISCUSSION

Studies of the adrenal during pregnancy, during estrous periods, after administration of toxic substances, and in the present type of investigation indicate that the mechanism of hypertrophy and increased weight might be effected by a similar mode of action upon the adrenal cortex. Evidence that the pituitary is responsible for cor-

tical hypertrophy has accumulated (Swann, 1940; Tepperman, Engel and Long, 1943). That such hypertrophy means increased cortical secretion has not yet been demonstrated. In the present study the increment of cell-size rather than hyperplasia appears to be responsible for the increased size of the cortex. The cellular hypertrophy was correlated with enlarged microliposomes and an increase in the number of macroliposomes.

Hoerr (1932) studying regeneration in the adrenal cortex concluded that cells were continually dying off in the reticular zone and being replaced by cells produced in the outer levels of the cortex. He concluded that the light and dark cells of the reticularis represented degenerating cortical cells in the last stage of senescence. This concept of centripetal migration of adrenal cortical cells has been supported by the study of Wotton and Zwemer (1943). In line with this concept Bennett (1939) utilizing histochemical reactions for detection of cortical hormones, designated the sub-capsular cortex as a pre-secretory zone, the outer portion of the fasciculata as secretory zone, and the portion of the cortex between the secretory zone and medulla as post-secretory zone which is characterized by the presence of numerous degenerating senescent cells. The increase noted in the frequency of necrotic cells in the enlarged reticularis zone following estrogen administration, is similar to the action of a toxic substance. In Hoerr's work, wherein guinea pigs were subjected to noxious gases and other toxic chemicals, similar increase in number of pycnotic cells was noted in the reticularis zone.

The increase in the dark-celled reticularis of estrogen-injected animals suggests the possibility of toxic effects of estrogen compounds. It has been found that estrogens are toxic to adrenalectomized rats and ferrets (Gaunt and Hays, 1938; Gaunt, Potts and Loomis, 1938; Cavanaugh and Gaunt, 1937; Schacher, Browne and Selye, 1937). The toxic effect of estrogen upon the cortical cells may stimulate compensatory secretion of the adrenocorticotropic hormone resulting in hypertrophy of the cortex.

Progesterone is known to be life-maintaining in adrenalectomized animals (Gaunt and Hays, 1938; Anderson and Kennedy, 1933; Greene, Wells and Ivy, 1939; D'Amour and D'Amour, 1937; Collings, 1939). This is suggestive in part for the differences observed in the effects between estrogen and progesterone on the adrenal cortical zones of the spayed female guinea pig. Histologically the administration of progesterone did not decrease the size of the fascicular zone of the adrenal cortex.

SUMMARY

The injection of moderate amounts of estrogen into spayed female guinea pigs for a short period of time instigated hypertrophy of the adrenal cortex with an increase of relative adrenal weight over that of the normal and castrated controls. Histological study revealed

that cortical hypertrophy was due to an increase in cell size in the fascicular and glomerulosar zones. Planimeter measurements were made of cross-sections through all glands. In the estrogen series the reticular zone of degenerate cells was greatly increased in size, the fascicular zone was reduced, while the glomerulosar zone was increased as compared to the controls.

A review of the recent literature indicates that the hypertrophy may be due to mediation of the anterior pituitary. In view of studies indicating differential activities of the cortical zones, the suggestion is made that estrogen causes accelerated degeneration of the cortical cells. This, in turn, stimulates compensatory secretion of an adrenocorticotropic factor to produce hypertrophy of the secretory cortical cells.

The injection of moderate amounts of progesterone for short periods of time caused an increase in relative adrenal weight and an enlargement of the glomerulosar and reticularis zones. Histologically the cortical cells showed an increment of size but otherwise resembled the controls. The life-maintaining properties of progesterone and the toxicity of estrogens are reflected in this study by the differential adrenal responses to injection of these two sex hormones.

REFERENCES

ALLEN, B. M., AND H. BERN: *Endocrinology* 31: 586. 1942.
ANDERSON, D. H.: *J. Physiol.* 83: 15. 1934.
ANDERSON, D. H., AND H. S. KENNEDY: *J. Physiol.* 76: 247. 1932.
ANDERSON, D. H., AND H. S. KENNEDY: *J. Physiol.* 77: 159. 1933.
BENNETT, H. S.: *Proc. Soc. Exper. Biol. & Med.* 42: 786. 1939.
BOLING, J. L., W. C. YOUNG AND E. DEMPSEY: *Endocrinology* 23: 182. 1938.
CAVANAUGH, C. J., AND R. GAUNT: *Proc. Soc. Exper. Biol. & Med.* 37: 226. 1937.
CLAUSEN, H.: *Endocrinology* 27: 989. 1940.
COLLINGS, W. D.: *Proc. Soc. Exper. Biol. & Med.* 40: 679. 1939.
COLLINS, V., J. L. BOLING, E. DEMPSEY AND W. C. YOUNG: *Endocrinology* 23: 188. 1938.
DANNER, M.: *Klin. Wchnschr.* 17: 658. 1938.
D'AMOUR, M. C., AND F. E. D'AMOUR: *Proc. Soc. Exper. Biol. & Med.* 40: 417. 1937.
DEANE, H. W., AND R. O. GREEP: *Am. J. Anat.* 79: 117. 1946.
ELLISON, E., AND J. BIRCH: *Endocrinology* 20: 746. 1936.
GAUNT, R., AND H. HAYS: *Am. J. Physiol.* 124: 767. 1938.
GAUNT, R., H. POTTS AND E. LOOMIS: *Endocrinology* 23: 216. 1938.
GREENE, R. R., J. WELLS AND A. C. IVY: *Proc. Soc. Exp. Biol. & Med.* 40: 83. 1939.
HOERR, N.: *Am. J. Anat.* 48: 139. 1932.
SCHACHER, J., J. S. L. BROWNE AND H. SELYE: *Proc. Soc. Exper. Biol. & Med.* 36: 488. 1937.
SELYE, H.: *J. A. M. A.* 115: 226. 1940.
SELYE, H., J. S. L. BROWNE AND J. B. COLLIP: *Proc. Soc. Exper. Biol. & Med.* 34: 472. 1936.
SWANN, H.: *Physiol. Rev.* 20: 493. 1940.
TEPPERMAN, J., F. ENGEL AND C. N. H. LONG: *Endocrinology* 32: 373. 1943.
WOTTON, R. M., AND R. L. ZWEMER: *Anat. Rec.* 86: 409. 1943.
ZALESKY, M.: *Anat. Rec.* 60: 291. 1934.
ZUCKERMAN, S., G. BOURNE AND D. LEWES: *Nature* 142: 754. 1938.

GONADOTROPIC MODIFYING ACTION OF SERA OF ANIMALS TREATED WITH HYPOPHYSEAL EXTRACTS

PHILIP A. KATZMAN, N. J. WADE AND EDWARD A. DOISY
From the Laboratories of Biology and Biological Chemistry, St. Louis University
ST. LOUIS, MISSOURI

THE PRESENT communication concludes our study of the effects produced by the chronic treatment of animals with implants or extracts of pituitaries of the same species. In our last publication on this topic (Katzman, Wade and Doisy, 1939), we reported that the sera obtained from sheep during 262 days of treatment with sheep pituitary extract continued to exhibit progonadotropic activity when tested with the same pituitary extract in immature female rats. In no instance during this period did this serum manifest inhibitory activity. Data were also presented showing that the sera of pigs treated for as long as 364 days with pig pituitary extract failed to exhibit consistent augmentatory or inhibitory properties.

At this time we wish to present the data which we have obtained from a continuation of the above studies. The animals Sh 2 (ewe), Sh 3 (ram), Sh 4 (ram) and Pg 2 (sow) together with the manner in which they were treated and the procedure used for assaying the serum in immature rats injected with pituitary extract have been described in detail in a previous publication (Katzman, Wade and Doisy, 1939). The treatment of sheep Sh 2 and Sh 3 with sheep pituitary extract, which during the first 262 days of the experiment produced progonadotropic sera (Katzman, Wade and Doisy, 1939) was carried on for an additional period of 388 days. The administration of pig pituitary extract to sheep Sh 4 and pig Pg 2 which for the first 140 days failed to produce definite augmentatory or inhibitory effects (Katzman, Wade and Doisy, 1939) was extended to 491 and 414 days, respectively.

PRO- AND ANTI-GONADOTROPIC EFFECTS OF SERA FROM SHEEP INJECTED WITH SHEEP PITUITARY EXTRACT

The serum of sheep Sh 3 began to exhibit inhibitory activity after 374 days of treatment[1] (table 1). At first this could be demonstrated

Received for publication April 26, 1947.

[1] The appearance of inhibitory activity was manifested first by the ovaries of the assay animals before a similar response was shown by the uteri. Thus, there was a transitory period during which the serum had an inhibitory effect on the ovaries and an augmentatory effect on the uteri. This might indicate that the LH and FSH of the pituitary extract were affected differently by the serum.

only with large doses (6.0 cc.), but with continued treatment progressively smaller amounts of serum produced this effect. Yet with still smaller quantities of the same serum a definite augmentatory response was evoked. Thus after 650 days of treatment, 0.6–1.5 cc. of serum was inhibitory but 0.06 cc. produced augmentation. Also, if a volume of the serum which produced an inhibitory effect in divided dosage was administered in a single injection at the time that the first injection of pituitary extract (given in the customary six doses) was made a progonadotropic rather than an inhibitory effect was obtained. For example, 1.5 cc. of the 650 day serum was inhibitory when administered in six injections but 2.0 cc. of the same serum produced augmentation when injected in one portion. Furthermore, if the single injection of serum was made as early as four days before the injections of the pituitary extract were begun the augmentation was further increased. This may indicate that the inhibitory principle is excreted or destroyed more rapidly than the substance responsible for the augmentatory response.

Attempts to separate the two activities of this serum by salting out, fractional precipitation with alcohol, treatment with acid, alkali or heat, and adsorption were unsuccessful.

The serum of another sheep (Sh 2) began to exhibit antigonadotropic properties after 297 days of treatment with the sheep pituitary extract.[2] With continued treatment the inhibitory power of the serum increased until by the 650th day 6.0 cc. of the serum given in six doses caused a depression of 50% in the response as measured by ovarian and uterine weights of the immature rats treated with the sheep pituitary extract. Contrary to the effect of serum of Sh 3, smaller doses or a single injection of this serum as well as pretreating the test animals failed to elicit a progonadotropic response. It may be of interest to point out that in the earlier part of the experiment the serum of this animal was not as strongly progonadotropic as was that of Sh 3.

EFFECT OF PROLONGED INJECTIONS OF SHEEP GONADOTROPIC HORMONE ON SEX GLANDS OF SH 2 AND SH 3

The testes of the ram Sh 3 after an initial rapid increase in size to 15 × 7.5 cm. (each testis) decreased to about 7 × 3 cm. by the end of the first year of treatment. After this time, however, the right testis began to increase in size until at the conclusion of the experiment it had again reached the previous maximum dimensions. The left testis was considerably smaller but was progressively increasing in size. Viable sperm were present in both testes.

---

[2] To conserve space detailed data on Sh 2, Sh 4 and Pg 2 have been omitted. The authors will gladly supply complete data on these experiments to any interested investigator.

After 340 days of treatment a hard mass was observed in the mammary gland of the ewe, Sh 2. At this time and continuing to the termination of the experiment considerable quantities of a cloudy, yellowish fluid could easily be expressed from both nipples. At autopsy the gland was found to be cystic and filled with an opaque fluid which

TABLE 1. GONADOTROPIC MODIFYING ACTION OF THE SERUM OF SHEEP, Sh 3, ON SHEEP PITUITARY EXTRACT IN IMMATURE FEMALE RATS

Day of treatment serum was obtained	Total quantity of serum injected in six doses*							
	6.0 cc.		1.5 cc.		0.6 cc.		0.06 cc.	
	Per cent deviation from controls†							
	Ovaries	Uterus	Ovaries	Uterus	Ovaries	Uterus	Ovaries	Uterus
297			+ 54.3	+ 65.5				
326			+ 31.7	+110.0				
352	+ 24.5	+172.0	+ 44.5	+ 83.5	+104.6	+200.0		
374	− 22.5	+ 35.5	+ 6.0	+ 91.0	+ 56.5	+ 87.0	+83.3	+99.5
385	− 22.0	+ 22.0‡			+ 25.5	+ 77.0	+72.5	+40.0
433	− 31.5‡	− 15.5‡	+ 5.4	+ 83.3	+ 38.4	+ 88.0	+14.2	+50.0
477	− 33.3‡	− 35.0‡			+ 11.0	+ 47.5	+13.7	+31.0
501	− 20.5	− 32.5	− 13.0	+ 33.3	+ 8.7	+ 13.0	+27.7	+37.0
554	− 21.2	− 27.7	− 12.8	+ 56.5	− 4.9	+ 72.0	+20.0	+51.0
594	− 25.8	− 42.8			− 28.5	− 4.0	− 0.3	− 1.0
650	− 51.0	− 43.0	− 42.5	− 10.0	− 4.0	− 1.0	+ 8.5	+39.0

	Total quantity of serum administered in a single injection*							
	2.0 cc.		1.0 cc.		0.5 cc.		0.25 cc.	
385	+110.0	+217.0	+ 25.0	+143.0	+ 30.4	+100.0	+30.0	+52.6
433			+ 52.0	+165.0	+ 39.5	+ 69.5	+47.3	+87.5
477			+ 57.6	+134.5				
501	+ 15.5	+ 66.0	+ 65.0§	+115.0§	+ 34.5	+ 82.5	+15.0§	+37.3§
		+ 77.3[a]	+ 77.3[b]	+ 77.5[a]				
			+ 85.7[b]	+116.0[b]				
			+113.0[c]	+107.0[c]				
554	+ 35.5	+ 92.5	+ 11.0	+196.0	+ 65.5	+ 82.5	+48.0	+21.3
594	+ 8.8	+ 62.5	− 3.6	+ 71.8	+ 20.5	+114.0	+25.6	+43.0
			+ 12.4[a]	+ 74.0[c]				
650	+ 21.0	+ 48.5	+ 4.0	+103.0	+ 73.5	+ 63.5	− 7.0	+ 5.5

* When the serum was administered in six doses, it was injected intraperitoneally twice daily for three days at the same time that the pituitary extract was injected subcutaneously. When the serum was given in a single dose the injection was made at the time of first injection of pituitary extract.

† Each value represents the deviation of the average weights of the respective organs of at least 5 experimental animals injected with serum and extract from that of 5 litter mate controls injected only with an equal amount of the same extract. The amount of extract used was such as to double or treble the ovarian weight. The deviation among the control groups was seldom as great as ±20%. However, somewhat smaller deviations may be regarded as being significant if they are consistently + or consistently −.

‡ Fifteen test animals used instead of the customary 5.

§ Ten test animals used instead of the customary 5.

[a] Serum injected 1 day before the first injection of pituitary extract.

[b] Serum injected 2 days before the first injection of pituitary extract.

[c] Serum injected 4 days before the first injection of pituitary extract.

gushed out on excision. The ovaries were small and the uterus infantile.

THE RESPONSE OF A SHEEP AND A PIG TO INJECTION WITH PIG PITUITARY EXTRACT

The treatment of the ram Sh 4 with pig pituitary extract was continued for 491 days.[2] At no time was there any indication that the considerably enlarged testes of this animal were decreasing in size. The serum of this animal in doses up to 6.0 cc. did not show consistent or significant inhibitory or augmentatory effects when tested with sheep pituitary extract in the immature female rats. When

tested with pig pituitary extract, the effect was usually inhibitory but this was not marked, the inhibition exceeding 20% in only a few instances.

The injection of the sow Pg 2 with pig pituitary extract was continued until the 414th day.[2] The serum of this animal after 170 days of treatment had in every instance an inhibitory effect on the gonadotropic action of the pig pituitary extract in the immature rats. However, even with the large doses (6.0 cc.) of serum the inhibition was not complete since in some test rats the uterine weight increase amounted to 50% and ovarian weight increase amounted to 26% of the weight increases of those organs of rats injected only with the gonadotropic extract.

TABLE 2. PROGONADOTROPIC RESPONSE IN IMMATURE MALE RATS

Serum		Sheep pituitary extract		Number of animals in group	Age (days)		Average testes weight (mg.)	Average seminal vesicle weight (mg.)	Average prostate weight (mg.)
cc.	Number of injections	Total dose mg.	Number of injections		At first injection	At autopsy			
0	0	112.5	30	5	21	37	738.0	34.8	76.9
7.5	30	112.5	30	5	21	37	825.4	47.8	92.0
7.5	30	0	0	5	21	37	787.6	20.3	32.7
0	0	0	0	5		37	787.5	14.2	41.8
0	0	150	10	5	21	27	453.2	10.6	34.8
10	10	150	10	5	21	27	458.6	17.5	43.8
0	0	0	0	5		27	340.6	7.0	21.7
0	0	150	10	5	21	27	508.5	10.4	36.5
5.0	10	150	10	5	21	27	589.4	18.3	48.8
0	0	0	0	5		27	379.3	7.8	22.5
5.0	10	0	0	5	21	27	351.6	7.5	21.0
0	0	0	0	5		27	315.1	6.6	24.4

PROGONADOTROPIC EFFECT OF SERUM IN IMMATURE MALE RATS

The progonadotropic serum of sheep Sh 3 was tested alone and with sheep pituitary extract in male rats (table 2). Twenty-one day old litter mates were used in each experiment. Serum was injected intraperitoneally and the sheep pituitary extract subcutaneously twice daily. The first group of animals was treated for 15 days and the others for 5 days. Autopsy was performed 24 hours following the last injections of serum and extract. The serum alone had little if any gonadotropic activity but it did significantly augment the effect of the pituitary extract especially as shown by the weights of the prostate and seminal vesicles. It should be pointed out that the failure of the serum alone to produce a gonadotropic effect furnishes additional evidence that the augmentatory effect is not due to luteinizing hormone (or ICSH) in the serum.

PROGONADOTROPIC EFFECT OF SERUM IN HYPOPHYSECTOMIZED IMMATURE FEMALE RATS

Rowlands and Williams (1940) have reported that progonadotropic activity in the serum of a goat treated with pig pituitary extract could be detected when the assay was conducted with intact female rats but that a positive response was not obtained when hypophysectomized rats were used as the test animals. The synergis-

TABLE 3. PROGONADOTROPIC RESPONSE IN HYPOPHYSECTOMIZED RATS

Age (Days)		Experimental animals		Litter mate controls†		
At operation	At first injection	Serum + pituitary extract		Pituitary extract alone		
		Ovaries mg.	Uterus mg.	Ovaries‡ mg.	Uterus mg.	
30	31	56.8	105.6	36.2	61.0	* 1.5 cc. (total) of serum administered in 6 injections at same time as pituitary extract.
30	31	69.4	127.2	44.0	47.0	
30	31	44.0	106.0	34.3	66.8	
31	31	63.8	83.6	30.3	69.2	
32	34	44.8	120.9	35.6	66.3	
32	34	54.3	117.4	32.3	65.0	
32	34	35.2	124.4	28.2	63.0	
Average		52.6	112.2	34.4	62.6	
35	39	52.3	97.6	35.4	46.9	* 1.0 cc. of serum administered in one injection at time of first injection of pituitary extract.
35	39	26.7	91.8	26.4	55.0	
35	39	78.0	103.3	44.5	38.8	
35	40	47.6	83.0	33.7	46.5	
35	40	45.3	75.2	38.4	44.2	
35	40	32.0	88.0	21.4	58.2	
35	40	65.5	85.7	41.2	37.2	
Average		49.6	89.2	34.4	46.7	

* Serum used was that obtained from sheep Sh 3 when it had been treated for 352 days with sheep pituitary extract.

† The litter mate controls which received pituitary extract alone were hypophysectomized and injected at the same time as the experimental animals which were injected with both serum and pituitary extract.

‡ The ovaries of untreated animals of these ages vary in weight from 14–20 mg.

tic substance which is produced in sheep by the administration of sheep pituitary extract does not behave in this manner. As shown in table 3, the sheep serum augmented the gonadotropic effect of sheep pituitary extract in hypophysectomized immature female rats. This was true whether the injections were started one day or five days after hypophysectomy. Litter mates, hypophysectomized on the same day,[3] were used in each case and the injections were carried out exactly as with the normal animals. In most instances the progonado-

[3] At autopsy all animals were examined carefully for remnants of the pituitary gland. Serial sections of the sella turcica were made in a number of instances including all those in which there was any doubt as to the completeness of the removal of the gland.

tropic response was as great in hypophysectomized as in normal animals.

Since the progonadotropic material produced in the sheep by injections of extracts of sheep pituitary glands is effective in hypophysectomized as well as in normal animals, it appears to be different from that evoked in the goat by extracts of pig pituitary glands as found by Rowlands and Williams (1940). It also differs from the progonadotropic sera obtained by Thompson (1937) from the horse and dog following a course of injections of sheep pituitary extract since alone it produces no gonadal stimulation in the intact immature female rat (Katzman, Wade and Doisy, 1939; Rowlands 1938).

DISCUSSION

In our earlier work (Katzman, Wade and Doisy, 1936, 1937 and 1939) we were unable to show that animals treated with extracts of the pituitaries of the same species develop substances inhibitory to the injected material. On the contrary, the sera of such animals augmented the gonadotropic activity of the hypophyseal extract. Similar observations were made by Rowlands (1938) and Thompson (1937). Collip (1937) found that the presence of progonadotropic activity in the serum was of short duration being replaced by inhibitory activity after only three months of treatment. The time required to produce inhibitory activity in the serum of our sheep by treatment with sheep pituitary extract indicates that this reaction toward pituitary material of the same species does not take place readily.

A comparison of the reaction of the gonads of ram Sh 3 and the gonadotropic-modifying properties of its serum during the course of the experiment offers some interesting findings. The testes increased in size rather rapidly during the first phase of the treatment with sheep pituitary extract. Then, in spite of the progonadotropic activity of the serum and the continued administration of the hypophyseal extract, involution set in. This was interpreted at the time as possibly indicating that insensitivity was developing to the injected gonadotropic material. After inhibitory activity did appear in the serum the testes began to increase in size and continued to increase in spite of the constantly increasing antigonadotropic titer of the serum. It is difficult to reconcile the findings in this animal with those of other investigators (Selye, Collip and Thompson, 1934; Fluhmann, 1936; Meyer and Gustus, 1935; Parkes and Rowlands, 1936; Rowlands, 1937; Severinghaus and Thompson, 1939; Kupperman, Meyer and Hertz, 1939; Thompson and Cushing, 1937; Collip, Selye and Williamson, 1938) who have reported that refractoriness induced by prolonged treatment with hypophyseal material or the injection of antigonadotropic serum results in atrophy and diminished function of the gonads.

It was demonstrated that the serum of sheep Sh 3 after more than

400 days of treatment was able both to enhance and diminish the gonadotropic effect of sheep pituitary extract in immature female rats depending upon the quantity of serum used or the time of injection. That serum obtained from animals treated with hypophyseal extracts may augment the effects of extracts of pituitaries of some species and inhibit the action of others has been observed by Rowlands (1938). The demonstration of both of these activities in the same serum toward the same pituitary extract using the same species for the assay is, to our knowledge, novel. It does not seem probable that these effects are produced by different quantities of a single substance since the progonadotropic effect exerted by the serum obtained during the first half of the treatment was proportional to the dose even when 6 cc. of serum were used. We believe progonadotropic activity could not be detected in the serum of sheep Sh 2 after it had acquired inhibitory properties because it contained a low titer of the former. If a single substance were responsible for these effects then it should have been possible to demonstrate augmentation with the serum of Sh 2 as well as with Sh 3.

The mechanism of the progonadotropic action of the serum of animals treated with extracts of the pituitaries of the same species remains entirely unsolved. That such serum does not contain known gonadotropic hormones is shown by its ineffectiveness to stimulate the gonads of immature female and male rats. Since hypophysectomized animals respond as well as normal controls, the action is not mediated by the hypophysis of the test animal as suggested by Rowlands and Williams (1940) for the progonadotropic activity which is induced in response to pituitary extract from a different species. The possibility remains that the augmentatory action may be due to the production of an anti-substance to some pituitary antagonist Evans et al., (1936) and Bunde and Hellbaum (1939) but there is no direct evidence available for this viewpoint.

SUMMARY

The serum of a ram after 374 days of treatment with sheep pituitary extract developed antigonadotropic properties. However, even after 650 days of this treatment progonadotropic activity could still be demonstrated to be present in the serum.

The serum of a ewe began to exhibit antigonadotropic activity after 297 days of injection of the sheep pituitary extract. Progonadotropic activity could not be demonstrated in the blood after this time.

The serum of a ram treated for 491 days with pig pituitary extract did not show consistent or significant inhibitory or augmentatory properties.

A pig treated with pig pituitary extracts developed gonadotropic inhibitory activity. This activity, however, was not very marked even after more than 400 days of treatment.

The progonadotropic activity of the sera of sheep treated with sheep pituitary extract could be demonstrated in immature male and hypophysectomized female rats.

The changes in the size of the testes of the animals receiving hypophyseal extracts for a long period of time could not be correlated with the progonadotropic or antigonadotropic titer of their sera.

ACKNOWLEDGMENTS

We are indebted to the Theelin Fund administered by the Committee on Grants for Research of St. Louis University for financial support and to Miss Corinne Dewes for technical assistance.

REFERENCES

BUNDE, C. A., AND A. A. HELLBAUM: *Am. J. Physiol.* 125: 290. 1939.
COLLIP, J. B.: *Canad. M. A. J.* 36: 199. 1937.
COLLIP, J. B.: *Tr. A. Am. Physicians* 52: 130. 1937.
COLLIP, J. B., H. SELYE AND J. E. WILLIAMSON: *Endocrinology* 23: 279. 1938.
EVANS, H. M., K. KORPI, R. I. PENCHARZ AND M. E. SIMPSON: *Univ. Calif. Publ. Anat.* 1: 237. 1936.
FLUHMANN. C. F.: *Proc. Soc. Exp. Biol. and Med.* 34: 691. 1936.
KATZMAN, P. A., N. J. WADE AND E. A. DOISY: *Endocrinology* 21: 1. 1937.
KATZMAN, P. A., N. J. WADE AND E. A. DOISY: *Endocrinology* 25: 554. 1939.
KATZMAN, P. A., N. J. WADE AND E. A. DOISY: *J. Biol. Chem.* 114: 56. 1936.
KUPPERMAN, H. S., R. K. MEYER AND R. HERTZ: *Endocrinology* 24: 115. 1939.
MEYER, R. K., AND E. L. GUSTUS: *Science* 81: 208. 1935.
PARKES, A. S. AND I. W. ROWLANDS: *J. Physiol.* 88: 305. 1936.
ROWLANDS, I. W.: *Proc. Roy. Soc. B*121: 517. 1937.
ROWLANDS, I. W.: *Proc. Roy. Soc. B*124: 492. 1938.
ROWLANDS, I. W., AND P. C. WILLIAMS: *J. Endocrinology* 2: 75. 1940.
SELYE, H., J. B. COLLIP and D. L. THOMPSON: *Proc. Soc. Exp. Biol. and Med.* 31: 566. 1934.
SEVERINGHAUS, A. E., AND K. W. THOMPSON: *Proc. Soc. Exp. Biol. and Med.* 40: 627. 1939.
THOMPSON, K. W.: *Proc. Soc. Exp. Biol. and Med.* 35: 634. 1937.
THOMPSON, K. W.: *Proc. Soc. Exp. Biol. and Med.* 35: 640. 1937.
THOMPSON, K. W., AND H. CUSHING: *Proc. Roy. Soc. B*121: 501. 1937.

SUCCINIC DEHYDROGENASE IN RAT OVARIAN TISSUES DURING PREGNANCY AND LACTATION[1,2]

ROLAND K. MEYER, S. W. SOUKUP, W. H. McSHAN, AND CLYDE BIDDULPH[3]

Department of Zoology, University of Wisconsin

MADISON, WISCONSIN

IT WAS SHOWN in a previous report from this laboratory (Meyer, McShan and Erway, 1945) that the ovaries of pregnant and pseudopregnant rats were high in succinic dehydrogenase (SDH-ase) and that this high activity was due to the lutein tissue rather than the other ovarian tissues. However, the determinations during pregnancy were made on days 18 and 20 of gestation, and therefore information is not available as to the way in which this enzyme changes in activity throughout pregnancy and lactation. Thus it was of interest to determine the SDH-ase activity of the corpora lutea and other ovarian tissues at intervals during these two phases of the reproductive cycle. The results obtained are presented in this report.

EXPERIMENTAL

Sprague-Dawley rats approximately 4 months of age were used in the study. Males were introduced into cages containing females on the day preceding the next estimated estrus and the females were examined on the following morning to determine whether insemination had taken place. The day following insemination was taken as the first day of pregnancy; the first day of lactation was considered as the one following parturition. The number of sucklings was reduced to 6 in all cases. Animals were killed on days 4, 7, 11, 15, and 20 of pregnancy and lactation.

The ovaries were removed immediately after the rats were killed by decapitation and the corpora were dissected out as rapidly as possible from the remaining ovarian tissue (ovarian residue). Then each kind of tissue was placed in a moist chamber surrounded by ice. During lactation the corpora of pregnancy were distinguished from those of lactation as the latter were lighter in color, had more prominent ovulatory papillae and were larger after day 7 of lactation than those of pregnancy. It was necessary to use the ovaries

Received for publication May 15, 1947.

[1] Aided in part by grants from the Wisconsin Alumni Research Foundation and Committee on Research in Endocrinology of the National Research Council.

[2] The data presented in this report were summarized in part in a paper given at the Conference on Menstruation and Its Disorders which was sponsored by the National Committee on Maternal Health, and held in New York, January 24 and 25, 1947.

[3] Present address: Utah State Agricultural College, Logan, Utah.

from at least 2 rats to obtain sufficient lutein tissue for the determination of SDH-ase during pregnancy and as many as 6 during lactation. The corpora lutea and the ovarian residue were combined from all rats killed on any particular day of pregnancy or lactation and weighed. The corpora were counted and their average weight for any given day was calculated from the total weight of the corpora.

Fifteen determinations were made of the percentage dry weight for each kind of tissue during the course of the study. The individual dry weight determinations ranged from 21.0 to 25.8 per cent with a mean of 22.8 for corpora lutea of pregnancy; from 16.0 to 25.0 per cent with a mean of 21.4 for the corpora of lactation; and from 18.6 to 26.0 per cent with a mean of 22.3 for ovarian residue. The dry weight values were in most cases quite constant, and the wide ranges are due to one or two determinations. The mean value for each tissue was used throughout in calculating the QO_2.

The tissues were prepared for the SDH-ase determinations by homogenizing them according to the method of Potter and Elvehjem (1936). The ovarian residue or lutein tissue was placed in a homogenizing tube with 0.1 ml. of glass distilled water and homogenized immediately. Sufficient water was added to make a 5 per cent homogenate and usually 0.2 ml. was used in the flask of a conventional Warburg apparatus that contained fortified substrate.

The fortified substrate solution consisted of 1 ml. of 0.1 M phosphate buffer of pH 7.4, 0.3 ml. 0.5 M sodium succinate, 0.2 ml. 3×10^{-4} M cytochrome c, 0.3 ml. each of 0.004 M $CaCl_2$ and $AlCl_3$ plus sufficient water to make a total volume of 3 ml. per flask (Potter, 1941; Potter and Schneider, 1942). The center cups in the Warburg flasks contained 0.1 ml. of 20 per cent potassium hydroxide. Approximately 20 minutes were required from the time the tissue was removed from the animal until it was placed in the water bath which was maintained at 38° C.

The same procedures were used in determining the cytochrome oxidase activity of the ovarian tissues except 0.3 ml. 0.114 M solution of Merck ascorbic acid was used as substrate with 0.3 ml. 9×10^{-4} M cytochrome c. The amount of tissue used was 0.1 ml. of a 2.5 per cent homogenate.

The contents of the flasks were allowed 10 minutes to come to equilibrium with the temperature of the water bath after which readings were made at 10 minute intervals for 1 hour. The SDH-ase and cytochrome oxidase activities are reported in terms of QO_2, i.e., the cubic milliliters of oxygen taken up per milligram of dry tissue per hour. These results are based on the average value for the first four 10 minute periods.

The cytochrome c was prepared from beef heart muscle according to the method of Keilin and Hartree (1937), except the final product was dialyzed against distilled water instead of 1 per cent sodium chloride solution (Potter 1941). The concentration of cytochrome c was determined by use of the Evelyn photoelectric colorimeter (Umbreit, Burris and Stauffer, 1945).

It was shown in an earlier report that 0.15 ml. 3×10^{-4} M cytochrome c was sufficient to elicit the maximum SDH-ase activity of ovarian tissue. However, 0.2 ml. of cytochrome c was used in the experiments to be sure that the concentration was sufficiently great in all cases. In a previous report it has been shown that the SDH-ase activity of homogenates of ovaries from pregnant rats is directly proportional to the amount of homogenate used (Meyer, McShan and Erway, 1945).

RESULTS AND DISCUSSION

The SDH-ase activity of the ovarian residue throughout pregnancy and lactation, and of the corpora of pregnancy and lactation, together with their weights are recorded in Table 1, and graphically presented in Fig. 1. The cytochrome oxidase activity for the corpora of diestrus, and of the 11th, 15th and 20th days of pregnancy and the ovarian residue on these days is presented in Table 2.

The average weights of the corpora of pregnancy during pregnancy and lactation and of the corpora of lactation are almost identical with those obtained in a study of the ATP-ase activity of

TABLE 1. SUCCINIC DEHYDROGENASE ACTIVITY OF LUTEIN TISSUE AND OVARIAN RESIDUE AND WEIGHT OF CORPUS LUTEUM DURING PREGNANCY AND LACTATION

Stage	No. of expts.	Succinic dehydrogenase activity (QO_2)			Weight (mg.) Corpora of:	
		Corpora of:		Ovarian residue		
		Pregnancy	Lactation		Pregnancy	Lact.
Pregnancy days						
4	2	20.8 (20.6–21.0)*	—	15.1 (14.3–16.0)	1.45 (1.4 –1.5)	—
7	2	33.9 (31.4–36.5)	—	18.6 (13.1–24.1)	1.6 (1.4 –1.9)	—
11	4	42.8 (38.5–53.0)	—	15.9 (15.4–16.3)	2.1 (1.9 –2.2)	—
15	3	42.3 (40.8–45.7)	—	13.9 (9.4–16.3)	4.2 (3.9 –4.6)	—
20	4	41.5 (40.2–42.9)	—	18.8 (15.5–22.5)	4.4 (4.3 –4.6)	—
Lactation days						
4	4	16.0 (11.9–19.9)	18.8 (12.5–25.4)	8.7 (5.9–19.4)	2.5 (2.4 –2.9)	1.5 (1.3–1.6)
7	5	21.8 (11.9–22.8)	26.6 (18.2–36.1)	13.9 (9.9–17.5)	1.5 (1.1 –1.9)	1.8 (1.3–2.1)
11	4	15.5 (5.2–23.4)	46.6 (35.6–60.9)	15.2 (9.3–24.2)	.84 (.7 – .98)	1.8 (1.6–2.0)
15	3	15.0 (14.0–16.1)	45.3 (35.8–51.7)	10.3 (5.2–16.1)	.63 (.45– .72)	2.0 (—)
20	4	20.2 (18.3–22.2)	63.3 (58.5–67.6)	9.9 (2.2–15.1)	.53 (.45– .58)	2.0 (1.8–2.2)

* Figures in parentheses are ranges.

the corpora during pregnancy and lactation (Biddulph, Meyer and McShan, 1946a, b). The corpora lutea do not show much increase in weight until the 11th day of pregnancy. Between the 11th and 15th days the weight increase is great, reaching its maximum on the 20th day of pregnancy. After parturition the weight of the corpora of pregnancy decreases rapidly until the 11th day of lactation after which there is only a slight decrease. The average weight of the corpora of lactation increases relatively little during lactation, reaching a maximum size on the 20th day of this period.

The SDH-ase activity of the corpora of pregnancy increases markedly from the 7th to the 11th day of gestation. From day 11 to 20 the activity is unchanged. Following parturition the enzyme decreases greatly by the 4th day of lactation. A relatively slight rise is seen on day 7 after which it falls to the lowest level on days 11 and 15, with a subsequent slight increase by day 20. The activity of

the corpora of pregnancy on day 20 are almost identical with those reported in a previous study of SDH-ase of the corpora of pregnancy on days 18 and 20 of pregnancy. The values for the corpora of pregnancy on the 4th day of gestation are approximately the same as those found for the corpora of diestrus as reported previously (Meyer, McShan and Erway, 1945).

The SDH-ase activity of the ovarian tissue remaining after the corpora have been removed shows relatively little variation when compared with the corpora of pregnancy and lactation. The greatest

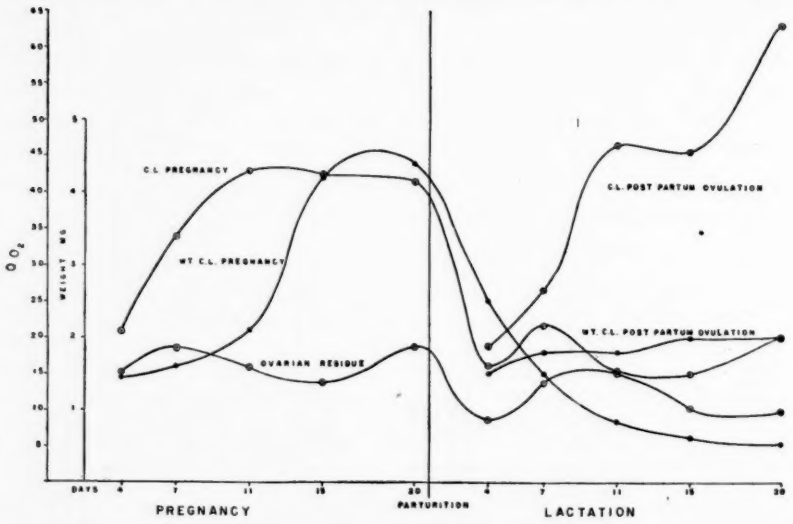


FIG. 1. The SDH-ase and weight of the corpora lutea of pregnancy during pregnancy and lactation, the SDH-ase and weight of the corpora of the post-partum ovulation during lactation, and the SDH-ase of the ovarian residue during pregnancy and lactation.

change in activity occurs immediately after parturition at which time the activity of the corpora of the post partum ovulation and of pregnancy are the lowest. The SDH-ase of ovarian residue increases at day 7 of lactation and decreases at day 15, remaining constant to the 20th day of lactation. It is difficult to determine whether these small variations in enzyme activity are of significance as far as function is concerned particularly since the ovarian residue is composed of more than one kind of endocrine tissue.

The cytochrome oxidase activity of diestrous corpora is shown by a QO_2 value of 372 which increases to 523 by day 11 of pregnancy and then remains at this high level until day 20 of pregnancy (Table 2). This increase in the activity of cytochrome oxidase during the

early phase of gestation is similar to the increase in the SDH-ase. This is not unreasonable since it is known that these two enzymes are both part of the succinoxidase system. The fact that the activity of cytochrome oxidase is greater in lutein tissue than the activity of SDH-ase is the same as has been found for other tissues (Schneider and Potter, 1943). The increase in the activity of these enzymes is correlated with the function of the corpora during pregnancy, and this relationship will be discussed below for SDH-ase, since more data is available for this enzyme. The cytochrome oxidase activity of the ovarian residue during diestrus is approximately the same as that for diestrous corpora and does not change throughout pregnancy.

Since the corpora increase markedly in weight during the last

TABLE 2. CYTOCHROME OXIDASE ACTIVITY OF LUTEIN TISSUE AND OVARIAN RESIDUE DURING DIESTRUS AND PREGNANCY

Stage	No. of expts.	Cytochrome oxidase activity (QO_2)		Weight of corpora mg.
		Corpora	Ovarian* residue	
Diestrus:	1	372	351	1.0
Pregnancy days				
11	2	523	345	2.1
15	2	492	320	4.2
20	2	498	350	4.4

* These values are high as they were not corrected for the autoxidation of the ascorbic acid used as substrates, but the differences are in the same direction as are corrected values obtained later.

half of pregnancy, and since the ketosteroids, cholesterol, and SDH-ase are also at their maximum value at this time (Laquer and Koets, 1945) it is obvious that the total quantity of these substances per corpus luteum is much greater during late than early pregnancy. The graphs in Figure 2 were drawn from calculations made from the data for the progesterone content of the corpora of pregnancy as reported by Laquer and Koets (1945) and from the data in Table 1. The curves represent the percentage change in the weight in the ketosteroids calculated as progesterone, and in the SDH-ase of the corpora of pregnancy using the corpora of diestrus as the base for the calculations. The data for the corpora of diestrus were taken from the paper by Meyer, McShan and Erway (1945). These curves show that the weight, progesterone and SDH-ase increase simultaneously from day 11 to day 20 of pregnancy. However, the percentage change in progesterone and SDH-ase is much greater than that of the weight. Immediately after parturition the percentage change in progesterone and SDH-ase decreases very markedly by day 4 of lactation when compared to that of the weight.

UNIVERSITY OF MICHIGAN LIBRARIES

From the data just discussed it is obvious that the SDH-ase undergoes changes in concentration in the corpora of pregnancy and lactation. The question arises as to whether high enzyme content is correlated with high functional activity of the corpora. That the ovaries are necessary throughout gestation for normal pregnancy and parturition in the rat has been established by various investigators (Johnson and Challans, 1930; Harris, 1927; Parkes, 1938) and results

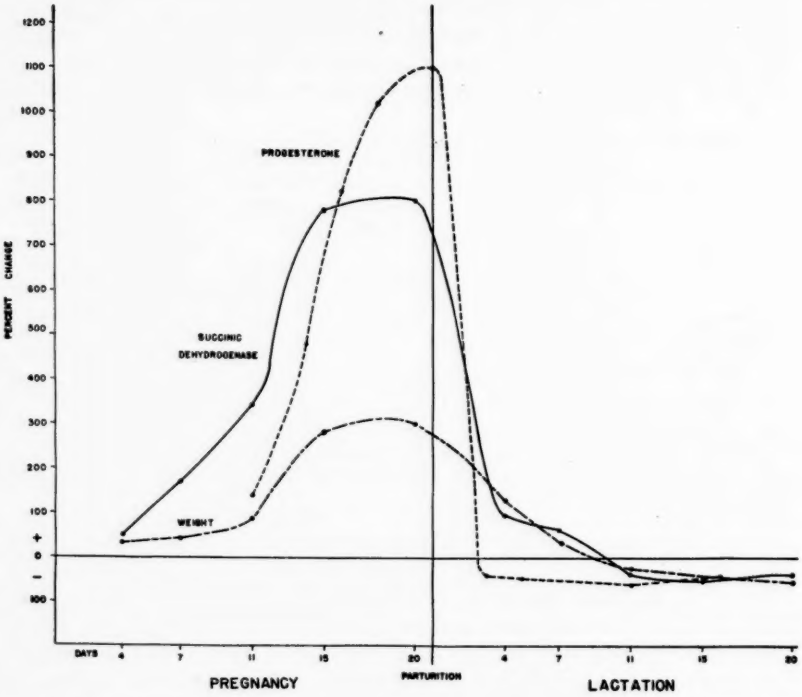


FIG. 2. Percentage change in the SDH-ase, weight and progesterone (ketosteroids) of the corpora lutea of pregnancy during pregnancy and lactation.

of ablation experiments clearly indicate that some lutein tissue must be present if pregnancy is to be maintained normally (Meyer and Roberts, unpublished). During lactation the corpora of post partum ovulation of the rat are functional since placentomata can be elicited throughout most of the suckling period (Lyon and Allen, 1938). Since the SDH-ase activity increases in the corpora of pregnancy and of lactation, it is apparent that the functional state of lutein tissue is correlated with the high enzyme activity. It seems reasonable to assume that SDH-ase is concerned in some way with the production and secretion of progesterone and perhaps other steroids which are necessary

for the maintenance of pregnancy and normal development of the embryos. Whether a higher level of enzyme activity at one stage in the life of the corpus when compared with another stage, means that function is greater when the enzyme is high as compared to when it is low is not readily ascertained. However, one approach to this problem is to analyze the relationship of enzyme concentration to specific functions of the tissue and to other enzyme systems. Some discussion of this problem has been presented in a recent publication by Potter (1946).

In the mouse, Atkinson and Hooker (1945) have obtained evidence which indicates that the amount of progesterone acting on the uterus is greater during the first half of pregnancy than during the latter half, and Atkinson and Leathem (1946) have shown in the mouse that the amount of progesterone affecting the uterus during lactation is constant throughout most of this period except during the first and last one or two days. If the criteria which they used actually measure the amount of progesterone being secreted by the corpora then it would appear that during the latter half of pregnancy relatively little progesterone is being secreted in the mouse, and also in the rat, if it is assumed that the situation is the same in the two species. Likewise during lactation the secretion of progesterone does not increase, but remains relatively constant after the early phase of lactation. (The SDH-ase increases progressively in the corpora of lactation reaching a very high value at the end of this period.)

The ketosteroids and cholesterol of the corpora of pregnancy in the rat are much greater during the last as compared to the first half of pregnancy as shown by Laquer and Koets (1945). It is also during the last half that the corpora of pregnancy reach their maximum size and weight. If these data are interpreted as meaning that the corpora are producing more hormones during the last half of pregnancy than during the first, then there is a disparity between this concept and the one based on the data of Atkinson and Hooker (1945). However, it may be that one or more factors in the latter half of pregnancy are preventing or inhibiting the characteristic action of progesterone on the uterus, and therefore all of the progesterone being secreted by the corpora may not be measured by use of this endpoint. In this connection Hooker (1945) has studied the effect of estrogen on the action of progesterone on the uterus of the mouse and, within the limits of the doses used, did not find any interfering effect.

Although the ketosteroids present in the corpora of pregnancy can be calculated as progesterone as Laquer and Koets (1945) have done, there is no proof that all of the ketosteroid fraction is progesterone. It is suggested that during the latter half of pregnancy the corpora are secreting other ketosteroids in addition to progesterone, i.e., adrenal cortical hormones, androgens and estrogens. Progester-

one itself is corticomimetic (Gaunt, Nelson and Loomis, 1938; Wells and Greene, 1939; Pfeiffer and Hooker, 1940) and the life of adrenalectomized rats can be maintained if gonadotrophins are administered to animals with ovaries (Emery and Schwabe, 1936; D'Amour and D'Amour, 1939; Fischer and Engle, 1939).

The adrenal cortical type of ketosteroid could well be of value in aiding the maternal tissues to meet the increased metabolic demands placed on them during the latter half of pregnancy and during parturition. This suggestion would put the corpus luteum during the latter half of pregnancy in the category of a supplementary adrenal cortex, and it may well be such to a lesser degree during the first half of pregnancy. Since the ketosteroids, cholesterol, weight and SDH-ase are at maximum values during the last half of pregnancy and decrease to lower or minimum values immediately or soon after parturition, it is suggested that the corpora of pregnancy are highly functional during the last half of gestation and may be more functional than during the first half. The much greater SDH-ase activity of the corpora of lactation as compared to those of pregnancy raises the question as to whether these corpora are more functional than those of pregnancy. This question is difficult to answer since information is not available concerning the ketosteroid content of the corpora during lactation. From the data of Atkinson and Hooker (1945) and Atkinson and Leathem (1946) it would seem that they are as active, but not more so, in secreting progesterone than the corpora in early pregnancy. However, the SDH-ase is much higher in the corpora during the latter part of lactation than that found during pregnancy, although the weight increases relatively little when compared to the last half of pregnancy. It would appear that the demands on the corpora of pregnancy are met by an increase in SDH-ase concentration during the first half of gestation and by an increase in the amount of tissue, having the same enzyme concentration, during the last half. During lactation the demand is met by an increase in enzyme concentration with very little increase in the amount of functional tissue. Thus it would seem that the corpora during the last half of pregnancy are producing other substances than progesterone. During lactation it is assumed that the corpora are mainly producing progesterone.

The rise in the SDH-ase content of the corpora during early pregnancy and in the corpora of lactation makes it of interest to determine what hormones might control the synthesis or increase the activity of the enzyme. During pregnancy it is logical to assume that the placenta and pituitary gland would be sources of such hormones as it has been shown that the lactogenic hormone of the pituitary gland and a luteotrophic factor in rat placenta will maintain the function of the corpora of the rat (Astwood and Greep, 1938). During lactation presumably the pituitary gland would be the only organ supplying

luteotrophin. It is indicated that the increase in SDH-ase during pregnancy is under the control of luteotrophins from the placenta and pituitary gland. However, unpublished data obtained in our laboratory demonstrate that the SDH-ase cannot be raised to as high a concentration as that found in the corpora of pregnancy and lactation by the administration of lactogenic preparations to hypophysectomized rats. We also have data which show that the corpora of hypophysectomized rats are not devoid of SDH-ase, having an enzyme activity like that of diestrous corpora.

Laquer and Koets (1945) have shown that the weight of the corpora in the rat and certain constituents including the lipids and protein can be increased to the values found in pregnancy by the administration of testosterone propionate to rats. Thus it seems that these changes can be elicited independently of the placenta. It is conceivable, however, that during pregnancy the placenta may indirectly influence the pituitary gland which in turn would act on the corpora.

SUMMARY

The SDH-ase of the corpora lutea of rats increases rapidly in concentration from day 4 to a maximum at day 11 of pregnancy, remains constant to day 20 and decreases rapidly to low levels after parturition. The weight of the corpora does not begin to increase rapidly until day 11, reaches a maximum between days 15 and 20 followed by a rapid decrease after parturition.

The SDH-ase of the corpora of the post partum ovulation increases in concentration from day 4 to a maximum at day 20 of lactation at which time the activity is greater than that found at any time for the corpora of pregnancy. The weight of the corpora of the post partum ovulation changes little during lactation. The SDH-ase activity of the ovarian residue varies little during pregnancy and lactation.

The cytochrome oxidase concentration was found to be much higher in the corpora at days 11 and 20 of pregnancy than during diestrus.

The changes in the concentration of succinic dehydrogenase are discussed in relation to the function of the corpora during pregnancy and lactation.

REFERENCES

ASTWOOD, E. B., AND R. O. GREEP: *Proc. Soc. Exp. Biol. Med.* 38: 713. 1938.
ATKINSON, W. B., AND C. W. HOOKER: *Anat. Rec.* 93: 75. 1945.
ATKINSON, W. B., AND J. H. LEATHEM: *Anat. Rec.* 95: 147. 1946.
BIDDULPH, C., R. K. MEYER AND W. H. MCSHAN: *Proc. Soc. Exp. Biol. Med.* 62: 36. 1946a.
BIDDULPH, C., R. K. MEYER AND W. H. MCSHAN: *Endocrinology* 38: 358. 1946b.
D'AMOUR, M. C., AND F. E. D'AMOUR: *Proc. Soc. Exp. Biol. Med.* 40: 417. 1939.
EMERY, F. E., AND E. L. SCHWABE: *Endocrinology* 20: 550. 1936.

FISCHER, A., AND M. ENGLE: *Lancet* 1: 354. 1939.
GAUNT, R., W. O. NELSON AND E. LOOMIS: *Proc. Soc. Exp. Biol. Med.* 39: 319. 1938.
HARRIS, R. G.: *Anat. Rec.* 37: 83. 1927.
HOOKER, C. W.: *Anat. Rec.* 93: 333. 1945.
JOHNSON, G. E., AND J. S. CHALLANS: *Anat. Rec.* 47: 300. 1930.
KEILIN, D., AND E. F. HARTREE: *Proc. Roy. Soc.* (*London*) B122: 298. 1937.
LAQUER, G. L., AND P. KOETS: *Proc. Soc. Exp. Biol. Med.* 60: 239. 1945.
LYON, R. A., AND W. M. ALLEN: *Amer. Jour. Physiol.* 122(3): 624. 1938.
MEYER, R. K., W. H. McSHAN AND W. F. ERWAY: *Endocrinology* 37: 431. 1945.
PARKES, A. S.: *J. Physiol.* 65: 341. 1928.
PFEIFFER, C. A., AND C. W. HOOKER: *Amer. Jour. Physiol.* 131: 441. 1940.
POTTER, V. R., AND C. A. ELVEHJEM: *J. Biol. Chem.* 114: 495. 1936.
POTTER, V. R.: *J. Biol. Chem.* 141: 775. 1941.
POTTER, V. R., AND W. C. SCHNEIDER: *J. Biol. Chem.* 142: 543. 1942.
POTTER, V. R.: *J. Biol. Chem.* 165: 311. 1946.
SCHNEIDER, W. C., AND V. R. POTTER: *J. Biol. Chem.* 149: 217. 1943.
UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER: Manometric Methods, p. 191. 1945.
WELLS, J. A., AND R. R. GREENE: *Endocrinology* 25: 183. 1939.

ACID AND ALKALINE PHOSPHATASES IN OVARIAN TISSUES OF THE RAT DURING PREGNANCY AND LACTATION[1,2]

ROBERT O. STAFFORD, W. H. McSHAN AND ROLAND K. MEYER
From the Department of Zoology, University of Wisconsin

UNIVERSITY OF WISCONSIN LIBRARIES

As PART of a program designed to study the interrelationships between hormones and certain intracellular enzymes, analyses of enzyme activities in ovarian tissues of the rat in different phases of the reproductive cycle have been made in this laboratory. The adenosinetriphosphatase activity of corpora lutea and the remaining tissues of the ovary has been studied (Biddulph, Meyer, and McShan 1946a, b) as well as the succinic dehydrogenase (Meyer, McShan, and Erway, 1945; Meyer, Soukup, McShan, and Biddulph, 1947).

The phosphatases are present in many tissues in varying quantity, and have been shown to be related directly to several tissue functions (Moog, 1946). Since Gomori (1941) and Corner (1944) showed by the use of histochemical methods that the corpora lutea of several species contain alkaline phosphatase, it was of interest to undertake a study to correlate the acid and alkaline phosphatase activities with the function of lutein and other ovarian tissues during pregnancy and lactation in the rat.

METHODS AND MATERIALS

With one exception, all previous methods for the determination of phosphatase activity in tissues have been histochemical, or have been based on the hydrolysis of various phosphate esters by tissue extracts. It has been demonstrated by Moog and Steinbach (1946) that much of the tissue phosphatase is bound to "heavy particles" of the cell, and that work on extracts must necessarily give values which do not represent the total enzyme activity of a tissue. It is desirable, therefore, to work with whole tissue homogenates rather than with extracts.

This was done by Moog (1946) who adapted the King-Armstrong method of phosphatase determination to homogenates of the developing chick embryo.

In the study to be reported here we made use of an adaptation of the method described by Huggins and Talalay (1945) which is based on the

Received for publication May 15, 1947.
[1] Aided in part by a grant from the Wisconsin Alumni Research Foundation.
[2] The data presented in this report were summarized in part in a paper given at the Conference on Menstruation and Its Disorders which was sponsored by the National Committee on Maternal Health, and held in New York, January 24, and 25, 1947.

colorimetric determination of free phenolphthalein liberated by the enzymatic hydrolysis of sodium phenolphthalein phosphate. This method has an advantage over the King-Armstrong method in that the number of manipulations required in the performance of the test is reduced.

By use of the Huggins-Talalay method, the degree of hydrolysis of an excess of substrate as indicated by the amount of *inorganic phosphate* liberated has been shown to be proportional to the concentration of enzyme, but the relationship between enzyme and *phenolphthalein* liberated is parabolic. This fact makes it impossible to express enzyme activity directly as the amount of phenolphthalein liberated. It is necessary to convert phenolphthalein liberated into arbitrary units.

Method. The test as modified was performed as follows: Specified quantities of tissue homogenate (Potter and Elvehjem, 1936) of known concentration were added to 50 ml. colorimeter tubes containing the properly buffered substrate and sufficient glass-distilled water so that total volume was constant in all tubes. The tubes had been brought to a temperature of 37° C. in a water bath.

The amount of substrate solution used was 2 ml. It was buffered at pH 5.4 with acetate buffer for the acid phosphatase (AcP-ase) and at pH 9.7 with barbiturate buffer for alkaline phosphatase (AlP-ase). One per cent homogenates were used throughout; quantities used were 0.2 ml. for the acid determinations and 0.15 ml. for the alkaline for corpora lutea, and 0.2 ml. both for acid and alkaline for ovarian residue, water being added to make a total volume of 2.6 ml. All determinations were made in triplicate.

The tissue-substrate mixture was incubated at a temperature of 37° C. for exactly one hour. The reaction was then stopped by the addition of 2.0 ml. of glycine buffer containing the phosphatase inhibitor sodium pyrophosphate. At the same time the test tubes were placed in a beaker of cracked ice to further insure sharp cessation of enzymatic action. The high alkalinity of the glycine buffer (pH 11.2) brought the pH of the entire mixture near the optimum for the development of the characteristic color of phenolphthalein.

All mixtures were then diluted to a volume of 10 ml. The tubes were wiped clean and dry, and the color was read in an Evelyn photoelectric colorimeter using a 540 filter. Although the color of the phenolphthalein does not change rapidly, all tubes were read exactly twenty minutes after addition of the glycine buffer to insure uniformity.

The small quantity of homogenate used in comparison with the final volume of the test mixture did not cause sufficient turbidity to warrant centrifuging. To correct for the slight cloudiness of the solutions, however, all tissue-containing tubes were read against a blank tube containing water, homogenate, and glycine buffer. To correct for the slight color developed by free phenolphthalein in the substrate, determinations were made each time on substrate blanks and these colorimeter L-values (log 100 minus log of the reading) were subtracted from those obtained for the tissue-containing tubes.

A straight line relationship was obtained by plotting known concentrations of phenolphthalein against the color (L-value) of these concentrations (Fig. 1). This standard phenolphthalein curve was used subsequently to convert L-values into quantities of phenolphthalein.

A phosphatase unit curve was then constructed as follows: Tissue homog-

enates of corpora lutea from rats on the 20th day of pregnancy were carried through the procedure described above in concentrations from 83.3 mg. tissue per 100 ml. to 500 mg. per 100 ml. These determinations were carried out in both acid and alkaline media and were repeated twice. The amounts of phenolphthalein liberated by the several concentrations of enzyme were determined from the standard curve (Fig. 1) and, expressed as mg. phenolphthalein per 100 ml. of homogenate, were plotted against mg. of tissue per 100 ml. of homogenate as shown in Fig. 2. Since it is more convenient to work

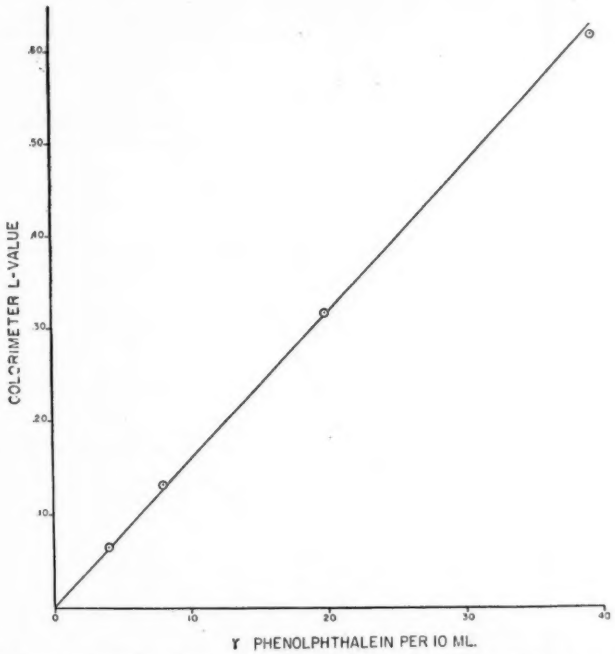


FIG. 1. Standard curve obtained with known concentrations of phenolphthalein.

with a straight line than with a parabolic curve, the values were plotted on log-log graph paper. The curves then appeared as shown in Fig. 3, Scale A.

The unitage is defined by Huggins and Talalay as follows: "10 units is the amount of enzyme which will liberate the colorimetric equivalent of 1 mg. of phenolphthalein from excess substrate in 1 hour at 37° under optimum conditions of pH." The scale of the x-axis of Fig. 3 was, therefore, rearranged (Scale B) so that 1 mg. phenolphthalein is equivalent to 10 phosphatase units. Since the curves obtained in acid and alkaline media are parallel, although quantitatively different, they demonstrate that the rate of hydrolysis of substrate for both enzymes is the same. A curve was drawn, therefore, parallel to these curves and passing through the reference point, i.e., 10 units equivalent to 1 mg. phenolphthalein. This is the unit curve.

When an L-value was obtained for a given sample, it was converted to gamma of phenolphthalein per 10 ml. of test solution by means of the standard curve, Fig. 1. This value was converted into mg. of phenolphthalein per 100 ml. of homogenate. The latter value was then applied to the unit curve (Fig. 3, Scale "B") and converted into phosphatase units per 100 ml. of homogenate. Since all values in this report are expressed as units per 100 mg. of fresh tissue, the next step was to calculate this value from units per 100

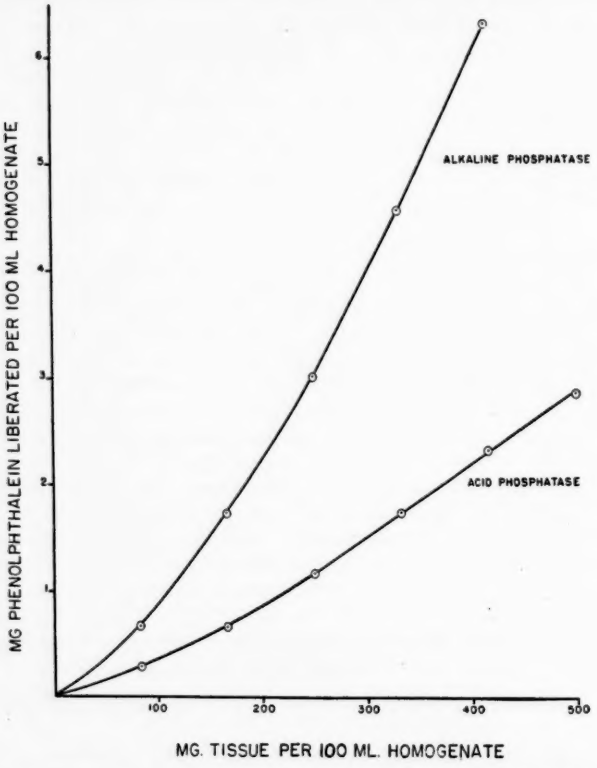


FIG. 2. Phenolphthalein liberated by increasing concentrations of lutein tissue homogenate in acid and alkaline media.

ml., inasmuch as the amount of tissue per 100 ml. was known.

Tissues studied. The animals used were virgin female rats of the Sprague-Dawley strain, three and one-half to four and one-half months old. The beginning of pregnancy was determined by the presence of sperm in the vaginal smears, the first day of pregnancy being counted as the day following the day on which sperm were observed. Upon becoming pregnant the rats were caged individually until they were killed. Litters were standardized at six young throughout lactation.

Animals were killed by decapitation on days 3, 6, 10, 14, and 19 of preg-

nancy, and on days 4, 7, 11, 15 and 20 of lactation. The proper corpora lutea were dissected from the ovaries, counted, and weighed as previously described (Biddulph, Meyer and McShan, 1946b). All discernible corpora lutea remaining were dissected free and discarded. The remaining ovarian tissue was weighed. The tissues to be analyzed were placed in 15 ml. homogenizing

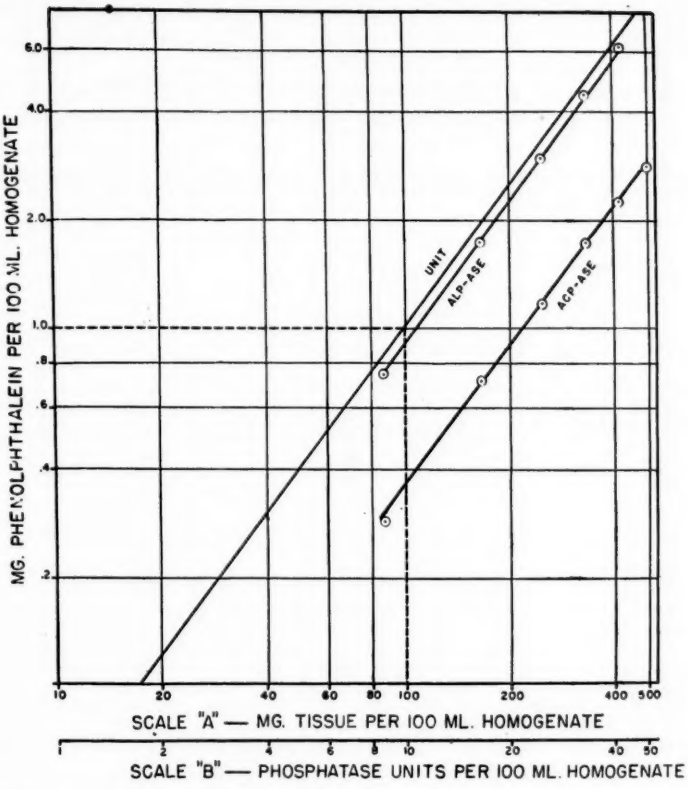


FIG. 3. Log-log plot of phenolphthalein liberated by increasing concentrations of lutein tissue homogenate in acid and alkaline media (Scale "A"), and phosphatase unit curve (Scale "B").

tubes containing 0.1 ml. of cold water, and were kept at the temperature of melting ice until homogenized and added to the test mixture.

Values for corpora of the second day of diestrus were determined to provide a base for the other values. Corpora of pregnancy were studied throughout gestation on the days indicated above and on day 4 of lactation. It was impractical to study these corpora farther into lactation, due to the large number of animals that would have been required for a single determination. The corpora lutea of the post-partum ovulation were similarly studied

throughout lactation. The ovarian residue was tested on all days indicated above.

Determinations were made on three rats for each stage of the periods studied except on the corpora of pregnancy on day 4 of lactation when only two rats were used. Each rat provided sufficient quantities of each of the tissues so that triplicate determinations could be made. Therefore the value for each of the tissues at any given stage is the mean of nine determinations.

RESULTS AND DISCUSSION

The acid and alkaline phosphatase values per 100 mg. of fresh lutein tissue and ovarian residue and the weights of the corpora are

TABLE 1. WEIGHT OF THE CORPUS LUTEUM AND ACID AND ALKALINE PHOSPHATASES OF LUTEIN AND OTHER OVARIAN TISSUES DURING PREGNANCY AND LACTATION

Stage	Wt. corpus luteum mg.	Phosphatase[1]			
		Acid		Alkaline	
		Lutein	Ov. tissue[2]	Lutein	Ov. tissue[2]
Diestrus	0.88	5.13 ± .65	2.81 ± .04	8.24 ± .53	9.73 ± .94
Pregnancy:					
3 days	1.64	3.54 ± .18	2.71 ± .25	5.40 ± .34	7.40 ± .99
6 days	1.81	3.52 ± .19	2.27 ± .22	8.24 ± .49	7.34 ± .27
10 days	1.90	3.49 ± .32	1.62 ± .07	8.47 ± .88	6.02 ± 1.08
14 days	4.21	4.92 ± .21	1.89 ± .08	13.00 ± 2.10	8.45 ± 1.86
19 days	5.17	4.50 ± .19	1.90 ± .15	12.87 ± .54	7.65 ± 2.05
Lactation:					
4 days	3.01[3]	4.75 ± .31	2.30 ± .19	15.41 ± .07	9.38 ± .37
	1.69[4]	3.35 ± .32	—	7.04 ± .86	—
7 days	2.00	3.69 ± .25	1.88 ± .07	11.88 ± .79	9.50 ± .53
11 days	2.01	4.01 ± .17	2.16 ± .10	10.75 ± .34	10.04 ± .16
15 days	1.87	4.54 ± .13	2.00 ± .16	13.40 ± .41	9.70 ± .31
20 days	2.10	4.61 ± .49	1.77 ± .15	9.96 ± 1.20	6.06 ± .74

[1] Expressed as phosphatase units per 100 mg. fresh tissue. Figures represent means of 9 determinations with standard errors.
[2] Represents ovarian tissues after removal of discernible corpora lutea.
[3] Values on this line are for corpora of pregnancy.
[4] Values on this and succeeding lines are for corpora of post partum ovulation.

shown in Table 1. The AcP-ase and AlP-ase for the two kinds of corpora and the weights of the corpora are presented graphically in Fig. 4. The corpora of early pregnancy show less activity in both enzymes than do those of diestrus. The AlP-ase activity begins to rise early in pregnancy, a marked increase occurring between days 3 and 6. After this initial rise there is no change for the following four days. Then, correlated with the rapid growth of the organ, another sharp rise in AlP-ase occurs. The activity then becomes stabilized until parturition, after which it again increases.

The AcP-ase shows practically no change during the first half of pregnancy. The small increase in the latter half which continues

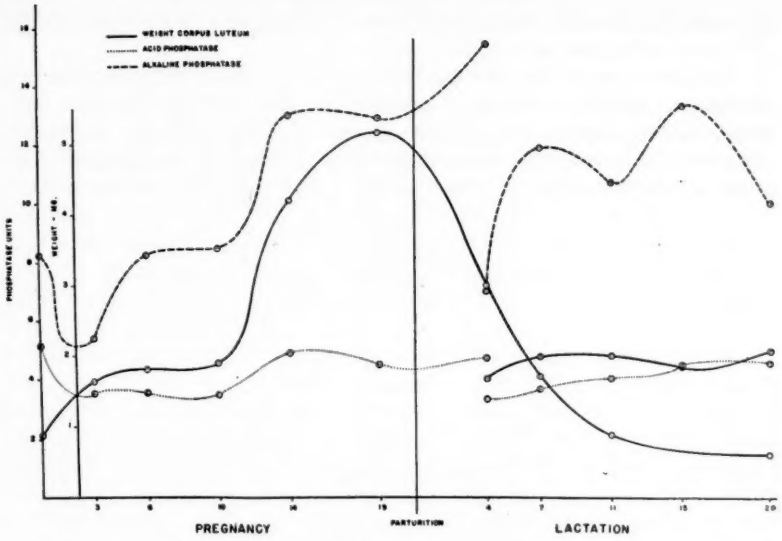


FIG. 4. The AlP-ase, AcP-ase, and weight of the corpora lutea of pregnancy through day 4 of lactation and of the corpora lutea of the post-partum ovulation during lactation. Phosphatase values are expressed as phosphatase units per 100 mg. of fresh tissue.

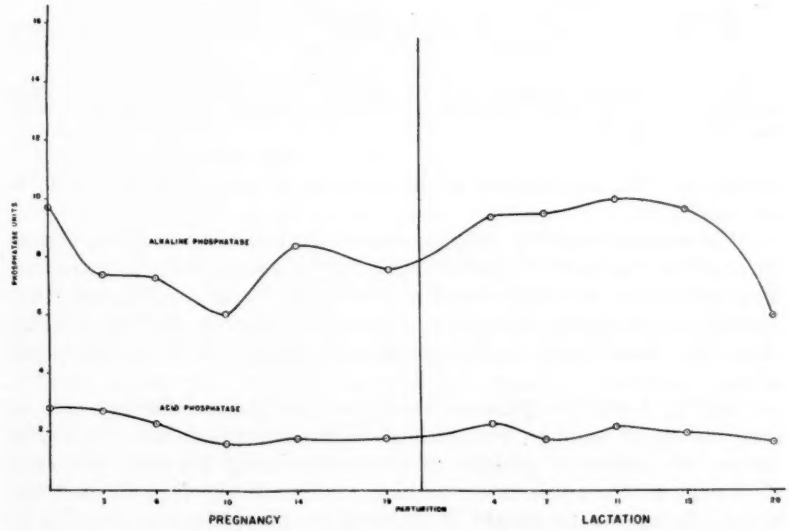


FIG. 5. The AlP-ase and AcP-ase of the ovarian residue during pregnancy and lactation.

through the fourth day of lactation is much less than that of the AlP-ase during the same time.

The corpora of the post-partum ovulation show no such marked changes in weight as those of pregnancy. The AcP-ase changes very little, rising slowly throughout lactation. The AlP-ase, however, rises rapidly in concentration between days 4 and 7, reaching a maximum on day 15. This is followed by a sharp decline between days

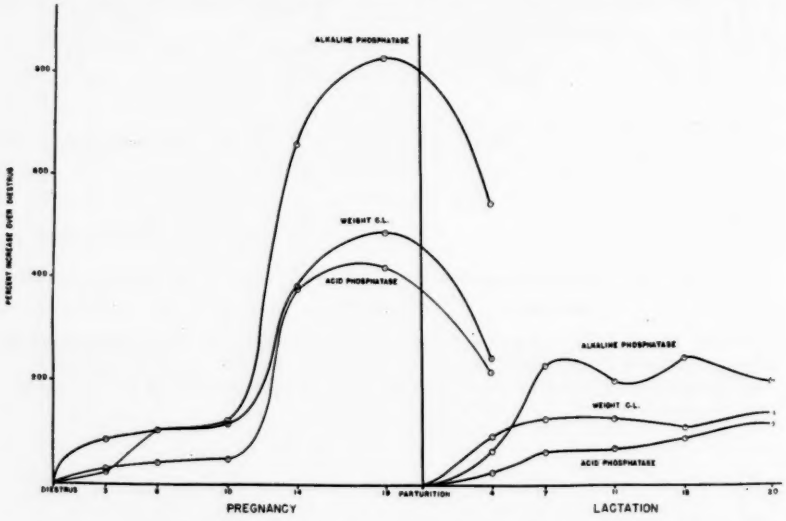


FIG. 6. Percentage change in AlP-ase, AcP-ase, and weight of the corpora lutea of pregnancy through day 4 of lactation and of the corpora of the post-partum ovulation during lactation.

15 and 20. The significance of the decrease between days 7 and 11 is not apparent.

The ovarian residue (Fig. 5) shows a much less variable course throughout the periods studied. The AcP-ase is practically constant. The AlP-ase on the other hand shows some changes worthy of note, namely the decreases on day 10 of pregnancy and on day 20 of lactation. The mean level during lactation is higher than during pregnancy.

In Fig. 6 the phosphatase values and weights of the two sets of corpora are graphed as percentages of the diestrus values *per corpus luteum* to present a picture of the relationship between the rate of change of the total amounts of enzyme activity present and the rate of change in the weight of the corpora. Since the number of cells in the corpus probably does not change appreciably throughout the growth and involution phases, these curves may be thought of as approximately depicting the changes within a single lutein cell.

One of the most significant points brought out by this method of presenting the data is the difference in the magnitude of change in the alkaline as compared to the acid phosphatase. In pregnancy the AcP-ase activity of the cell increases and declines approximately at the same rate as the weight, both maxima being between 400 and 500% of the corpora of diestrus. The AlP-ase at its maximum, however, increases to over 800% of the base value, increasing almost twice as much as the weight. The increase in AlP-ase is due both to an increase in concentration and to an increase in the amount of tissue present while the AcP-ase concentration remains almost constant, and the increase in the AcP-ase is due largely to the increase in the amount of tissue.

In lactation, where the weight does not increase significantly after the 7th day, there is a slight change in AcP-ase per corpus luteum due to the gradual increase in enzyme concentration rather than to an increase in the amount of tissue. The AlP-ase, as during pregnancy, increases at a greater rate per corpus luteum than the weight because of the large increase in concentration.

Comparison of the rate of change of these two enzymes, as well as the rate of change in the ATP-ase (Biddulph, Meyer and McShan 1946a, b) and succinic dehydrogenase (Meyer, Soukup, McShan and Biddulph, 1947) in the growing and regressing corpus luteum during pregnancy and lactation provides the basis for the following generalization:

In an endocrine gland the activity of an enzyme may or may not increase or decrease at the same rate as the gland grows or involutes.

From the discussion thus far it is obvious that the two phosphatases studied in this investigation do vary in concentration during the growth and involution of the corpus during pregnancy and lactation. The question logically arises, then, as to whether high enzyme activity can be correlated with a high level of function. This question has been discussed in previous papers dealing with the change in concentration of succinic dehydrogenase (Meyer, McShan and Erway, 1945; Meyer, Soukup, McShan and Biddulph, 1947) and of ATP-ase (Biddulph, Meyer and McShan, 1946a, b). The reader is referred particularly to the papers on succinic dehydrogenase which discuss the evidence for the concept that the corpus is as functional or perhaps more so during late as compared to early pregnancy. A part of the evidence supporting this idea was found in the high enzyme activity in the last half of pregnancy. The data reported in this paper also show that the acid and alkaline phosphatases are in high concentration during the last half of pregnancy as compared to the first.

The role played by the phosphatases in the function of the lutein cell must of necessity be purely speculative in the light of our present knowledge. However it is possible that AlP-ase is important in some reaction involved in the synthesis of luteal hormones. Little is known

about the processes involved in the production of progesterone, but it does not seem illogical to assume that one or more of the intermediate reactions involve phosphate transfer in which phosphatases would play a part.

It is also conceivable that phosphatases may play an important role in the secretion of steroids by the lutein cell since phosphate esterification of several substances is believed to be necessary for the passage of these substances across cell membranes (Moog, 1945). It seems reasonable that dephosphorylating enzymes such as those under consideration might be of importance in the passage of luteal hormone precursors into the lutein cell and the secretion of the finished product out of the cell.

SUMMARY

The Huggins-Talalay method for phosphatase determination was adapted to the determination of acid and alkaline phosphatases in the corpus luteum and corpus luteum free ovarian residue of the rat in diestrus, pregnancy, and lactation.

Acid phosphatase was present in lesser quantity than alkaline phosphatase at all times in all tissues studied.

The acid phosphatase activity per unit weight of the corpora of pregnancy rises slightly in mid-pregnancy, remaining at this level until after parturition. In the corpora of lactation, it rises slowly throughout the life of the corpora.

The alkaline phosphatase per unit weight of the corpora of pregnancy rises from its initial low level early in pregnancy and again between days 10 and 14, continuing to rise after parturition. The alkaline phosphatase rises in the corpora of lactation throughout the 15th day of lactation, falling markedly between days 15 and 20.

The non-lutein ovarian tissue presents unchanging acid phosphatase activity throughout the periods studied. The alkaline phosphatase activity of the ovarian residue is somewhat higher in lactation than in pregnancy, and drops markedly in the first half of pregnancy and toward the end of lactation.

REFERENCES

Biddulph, C., R. K. Meyer and W. H. McShan: *Proc. Soc. Exper. Biol. & Med.* 62: 36. 1946a.

Biddulph, C., R. K. Meyer and W. H. McShan: *Endocrinology* 38: 358. 1946b.

Corner, G. W.: *Science* 100: 270. 1944.

Gomori, G.: *J. Cell. & Comp. Physiol.* 17: 71. 1941.

Huggins, C., and P. Talalay: *J. Biol. Chem.* 159: 399. 1945.

Meyer, R. K., W. H. McShan and W. F. Erway: *Endocrinology* 37: 431. 1945.

Meyer, R. K., S. W. Soukup, W. H. McShan and C. Biddulph: *Endocrinology* 41: 35. 1947.

Moog, F.: *Biol. Rev.* 21: 41. 1946.

Moog, F. *J. Cell. & Comp. Physiol.* 28: 197. 1946.

Moog, F., and H. B. Steinbach: *J. Cell. & Comp. Physiol.* 28: 209. 1946.

Potter, V. R., and C. A. Elvehjem: *J. Biol. Chem.* 114: 495. 1936.

COMPARISON OF LIVER-GLYCOGEN DEPOSITION AND WORK PERFORMANCE TESTS FOR THE BIO-ASSAY OF ADRENAL CORTEX HORMONES

MARVIN L. PABST, RUTH SHEPPARD AND MARVIN H. KUIZENGA

From the Research Laboratories, The Upjohn Company

KALAMAZOO, MICHIGAN

FOR THE BIO-ASSAY of adrenal cortical hormone preparations the survival-growth test (Cartland and Kuizenga, 1936) is of value, since it is responsive to the composite activity of adrenal cortex extracts with respect to both the known compounds and amorphous fractions (Kuizenga, Nelson and Ingle, 1943; and Kuizenga, Nelson, Lyster and Ingle, 1945). Factors influencing electrolyte and carbohydrate metabolism cannot, however, be differentiated by this test. Responses obtained with the pure C-11-oxygenated steroids in the muscle work test, compared with the relative inactivity of desoxycorticosterone and the amorphous fractions by this test, led us to the conclusion that it could be used for the quantitative determination of the specific factors effective in carbohydrate metabolism (Ingle, 1944; and Ingle and Kuizenga, 1945). For clinical use, a knowledge of the content of these factors in adrenal cortical extracts is of importance (MacBryde and De La Balze, 1944; and Forsham, Thorn and Bergner, 1946). We have therefore employed the work test to obtain such information. The liver-glycogen deposition response in adrenalectomized rats has also been investigated for this purpose (Reinecke and Kendall, 1942; Bergman and Klein, 1943; and Olson, Jacobs, Richert, Thayer, Kopp and Wade, 1944). We have further investigated this bio-assay method and compared the results, quantitatively, with those obtained by the muscle work test. These results show that the order of activity for the four C-11-oxygenated steroids and two adrenal cortical extracts is the same by both methods.

METHODS

Liver-glycogen deposition method. The method used was similar to that described by Olson *et al.* (1944). Male rats of the Sprague-Dawley strain which weighed 140–160 gm. were used. The test was carried out on groups of 20–40 animals which were removed from the regular stock diet of Purina Dog Chow, anesthetized with ether and bilaterally adrenalectomized by the method of Ingle and Griffith (1942). They were placed in wire-bottom cages

Received for publication May 19, 1947.

and fed a high protein diet[1] and tap water containing one per cent sodium chloride until the morning of the fourth postoperative day. The diet was then removed from the cages, and on the morning of the fifth postoperative day the drinking water was removed and injections were started. Injections of crystalline hormones and cortical extracts in cottonseed oil were made subcutaneously in four divided doses at two-hour intervals. In order to keep at a minimum possible error due to absorption from varying amounts of oil, the concentrations were adjusted after preliminary observations so that the volume for each injection was not outside the limits of 0.18 cc. to 0.25 cc. Between $1\frac{1}{2}$ and $2\frac{1}{2}$ hours after the last injection, the animals were anesthetized with 0.75 cc. of two per cent solution of cyclopal sodium intraperitoneally, the abdomen was opened and the entire liver was removed for analysis.

Immediately after removal and blotting with absorbent paper to remove blood, the liver was placed in a tared flask containing 30 per cent KOH (approximately 2.0 cc. per gm. liver) and weighed. About thirty seconds elapsed from the time the abdomen was opened until the liver was submerged in the alkali. Complete digestion was accomplished by heating 30–40 minutes on the steam bath.

The alkaline liver digests were made up to 100-cc. volume and aliquots taken for glycogen determinations. The method of Good, Kramer and Somogyi (1933) was used with modifications. The glycogen precipitates were obtained by addition of absolute alcohol, heating to boiling on a water bath, cooling and heating as before and finally centrifuging. Hydrolysis of glycogen was accomplished by adding 2.5 cc. of normal H_2SO_4 to the glycogen in a centrifuge tube and autoclaving at 15 pounds of pressure for 15 minutes. After autoclaving, glucose determinations were made by the modified iodometric copper method of Shaffer and Somogyi (1933), using reagent 50 containing no potassium iodide. The iodide and the sulfuric acid were added just before titration with sodium thiosulphate. Glycogen values were expressed in terms of their glucose equivalents, and calculations expressed as per cent glycogen of fresh liver weight. Control experiments with known solutions of glucose, known solutions of glycogen and alkaline digests of liver, with known quantities of glycogen added, showed satisfactory recovery.

Muscle work test. Male rats of the Sprague-Dawley strain which weighed 180 ± 2 gm. were adrenalectomized and nephrectomized in a single-stage operation and immediately subjected to the work test. The apparatus and procedures were identical with those previously described (Ingle, 1944). Subcutaneous injections of the hormones were made at the beginning of stimulation and again six hours later. Stimulation was continued either until the muscle ceased to respond or for a period of twenty-four hours. The average total number of recorder revolutions for the twenty-four-hour period was used in the calculation of "units" from a previously established standard curve.

The four crystalline steroids (11-dehydrocorticosterone, corticosterone, 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone) were obtained from beef and hog adrenal concentrates (Kuizenga *et al.*, 1945).

[1] The diet was similar to the OG-2 diet of Olson, Jacobs, Richert, Thayer, Kopp and Wade (1944). It contained: commercial casein 55 per cent, dried brewer's yeast 6 per cent, U.S.P. salt mixture No. 1, 4 per cent, celluflour 2 per cent, calcium carbonate 1 per cent, cystine 0.3 per cent, sucrose 11.7 per cent, primex (vegetable shortening) 19 per cent, codliver oil 1 per cent and 2 mg. of choline chloride per gram of food.

The samples used were the purest that could be obtained by recrystallization from suitable solvents. According to melting point, specific rotation and chemical analysis, they were considered analytically pure. The two adrenal cortical extracts were prepared as previously described.

RESULTS

In a control study, adrenalectomized rats were subjected to the

TABLE 1. GLYCOGEN DEPOSITION OBTAINED WITH FIVE CRYSTALLINE HORMONES OF THE ADRENAL CORTEX, ONE EXTRACT OF BEEF AND ONE OF HOG ADRENAL IN FASTED, ADRENALECTOMIZED RATS

Preparation	No. rats	Total dose	Mean glycogen deposition %	Regression line coefficients*		Significance of difference in b. t†
				$a \pm \epsilon a$	$b \pm \epsilon b$	
11-dehydrocorticosterone	10	0.35 mg.	0.25 ± .03	1.29 ± .09	2.42 ± .27	1.88
	10	0.50 mg.	0.48 ± .05			
	10	0.71 mg.	0.87 ± .09			
	10	1.00 mg.	1.34 ± .08			
corticosterone	10	0.35 mg.	0.45 ± .07	1.28 ± .08	1.71 ± .24	0.50
	10	0.50 mg.	0.87 ± .08			
	10	0.71 mg.	0.95 ± .09			
	10	1.00 mg.	1.28 ± .07			
11-dehydro-17-hydroxycorticosterone	10	0.25 mg.	0.77 ± .07	1.79 ± .07	1.85 ± .14	0.00
	10	0.35 mg.	0.71 ± .09			
	10	0.50 mg.	1.37 ± .07			
	10	0.71 mg.	1.54 ± .10			
	10	1.00 mg.	1.74 ± .06			
17-hydroxycorticosterone	9	0.125 mg.	0.45 ± .07	2.18 ± .08	1.95 ± .12	0.50
	10	0.25 mg.	0.98 ± .09			
	10	0.35 mg.	1.25 ± .08			
	10	0.50 mg.	1.68 ± .08			
	10	0.71 mg.	1.82 ± .11			
	10	1.00 mg.	2.22 ± .14			
Desoxycorticosterone acetate	5	2.0 mg.	0.02			
Beef adrenal cortical extract	10	40 gm. gland	0.32 ± .08	0.32 ± .09	2.33 ± .34	1.26
	10	60 mg. gland	0.74 ± .10			
	20	80 gm. gland	1.02 ± .09			
Hog adrenal cortical extract	20	20 gm. gland	0.42 ± .06	1.11 ± .10	2.23 ± .19	1.61
	9	40 gm. gland	1.15 ± .10			
	10	80 mg. gland	1.76 ± .11			

* Glycogen deposition in per cent of fresh liver weight may be predicted from the logarithm of the dose by these regression line coefficients according to Fisher (1932); i.e., $y = a + bx$ or $y = \bar{y} + b(x - \bar{x})$.

y = per cent glycogen of fresh liver
x = logarithm of dose
$a = \bar{y} - b\bar{x}$
$b = \dfrac{\Sigma y(x - \bar{x})}{\Sigma(x - \bar{x})^2}$
$\bar{y}$ = mean value of y
$\bar{x}$ = mean value of d

The standard error of "a" is calculated by the formula $\sqrt{\dfrac{\Sigma d^2}{n(n-1)}}$; the error of "$b$" is from Irwin's formula $\epsilon^2 = \dfrac{\sigma^2}{\Sigma\left[n(x - \bar{x})^2\right]}$ where $\sigma = \sqrt{\dfrac{\Sigma d^2}{n-1}}$.

† t is computed according to Burn (1937) and evaluated according to Fisher.

conditions of the glycogen test and on the fifth postoperative day were injected subcutaneously with cottonseed oil. There were two groups of four rats each. One group received a total dose of 2.0 cc. per rat, and the average deposition of liver glycogen was 0.016 per cent. The other group received a total injected dose of 0.8 cc. per rat, and the average deposition of liver glycogen was 0.012 per cent. This indicates that only a trace of liver glycogen is present in the fasted adrenalectomized rat after subcutaneous injections of varying quan-

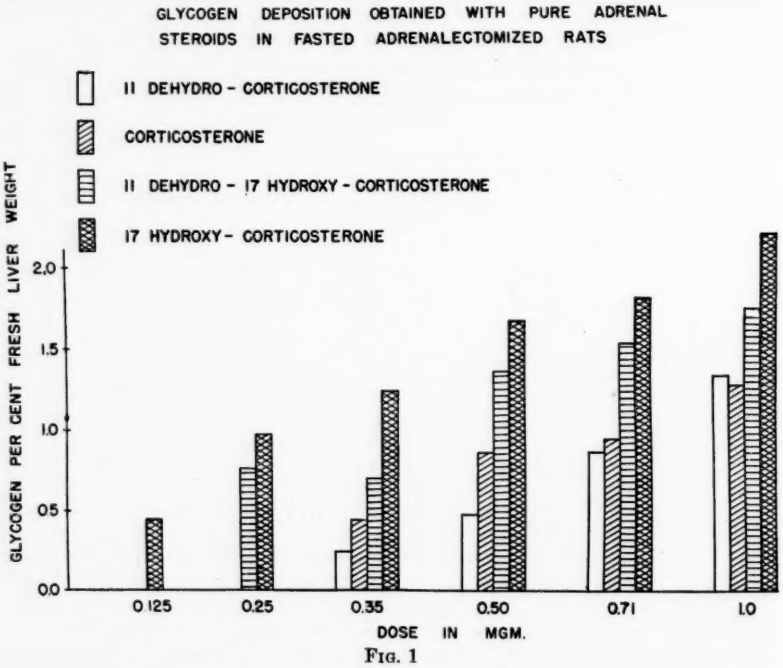


FIG. 1

tities of oil. Another control test on two groups of five rats each showed that the normal rat first fed and then fasted under the conditions of the test maintained an average liver glycogen of 0.40 per cent.

The pure steroids and extracts were tested by the glycogen method at three to six dosage levels, and the mean glycogen responses for each dose are given in Table 1. Desoxycorticosterone acetate was found to be inactive when administered in a dose of 2 mg. This failure of desoxycorticosterone to give a glycogen deposition response has repeatedly been observed by others (Olson, Jacobs, Richert, Thayer, Kopp and Wade, 1944; Reinecke and Kendall, 1943; and Dorfman, Shipley, Ross, Schiller and Horwitt, 1946). The relative potencies of the four C-11-oxygenated steroids are graphically shown in Figure 1.

It appears that 11-dehydrocorticosterone is the least active and that, in the order named, corticosterone, 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone are increasingly active, the latter having been found to be the most active of the group.

The difference in activity of the hog and beef adrenal extracts is shown in Figure 2. It is apparent that a given amount of hog adrenal tissue yields about twice the biological activity, as measured by the

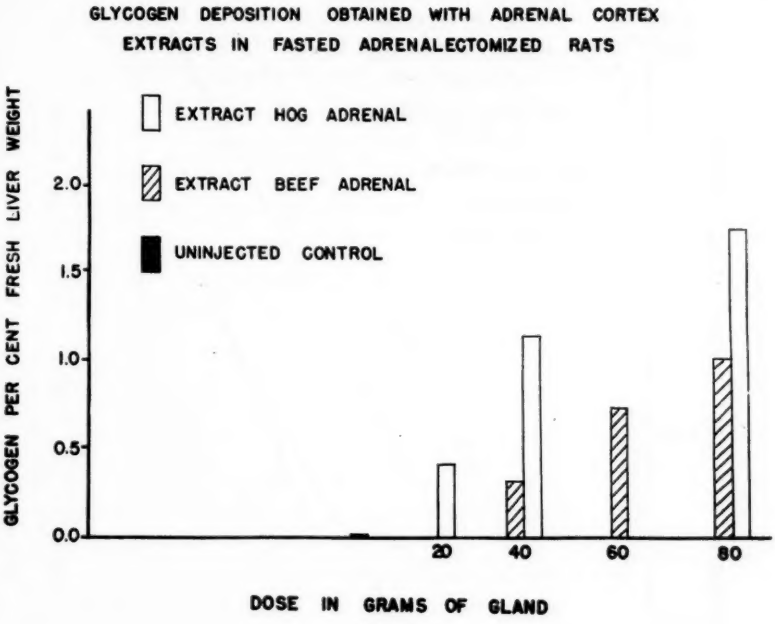


FIG. 2

glycogen test, that the same amount of beef adrenal tissue yields. It is interesting that approximately the same ratio of potency had previously been observed with other samples of the gland extracts, not only by the muscle work test but slso by the survival-growth test (Kuizenga, Wick, Ingle, Nelson and Cartland, 1943.)

In order to determine the accuracy of the liver-glycogen deposition method, the data recorded in Table 1 were submittid to statistical analysis and the logarithm dose-response regression lines and errors were calculated (Fisher, 1932; Irwin, 1937; and Burn, 1937). The lines for the pure steroids are given in Figure, 3, and for the the adrenal extracts in Figure 4. These responses relating glycogen depsition to log dose can be seen to be straight lines, and the variations around the lines are seen to be small. The slopes of the regression lines of the C-11-oxygenated steroids and the two adrenal extracts

do not differ significantly. Therefore, any one of the pure steroids may be used as a reference standard for the determination of biological activity. Since 11-dehydro-17-hydroxycorticosterone has quite generally been used by others in the study of adrenal cortex hormone

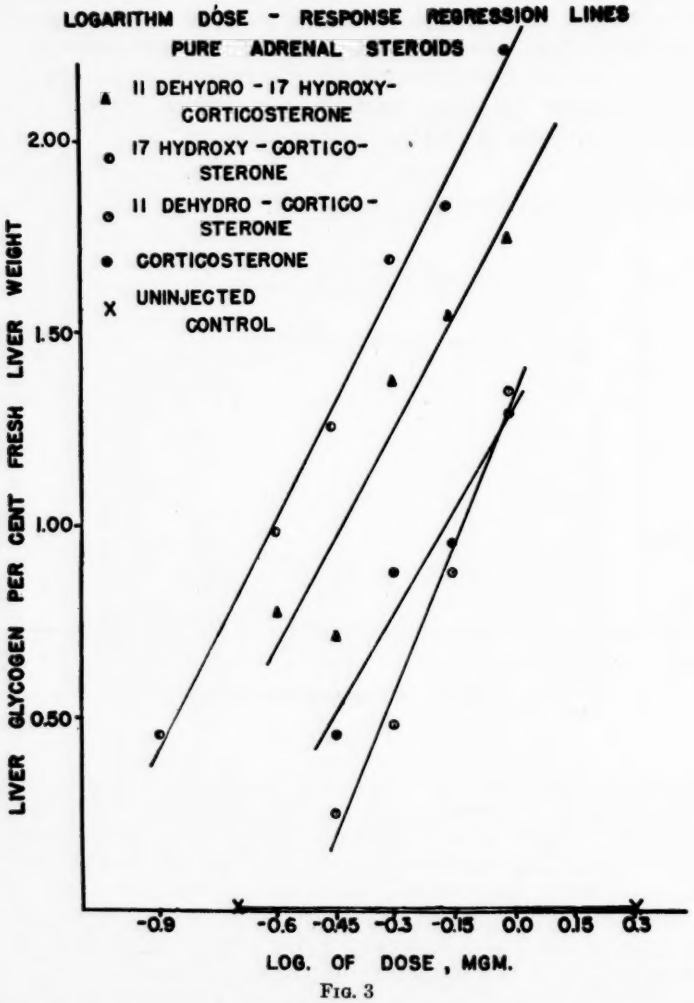


FIG. 3

assays, and since this compuond is the reference standard used in the muscle work test in this laboratory, we have determined the relative activity of the steroids by both the glycogen deposition and muscle work tests in terms of 11-dehydro-17-hydroxycorticosterone. These results, with the estimation of error, are given in Table 2. The work

test was applied, as previously described, where the error was determined to be ± 20 per cent (Ingle, 1944). The same relative order of activity is apparent by both methods.

The activity of the adrenal cortical extracts in terms of 11-dehy-

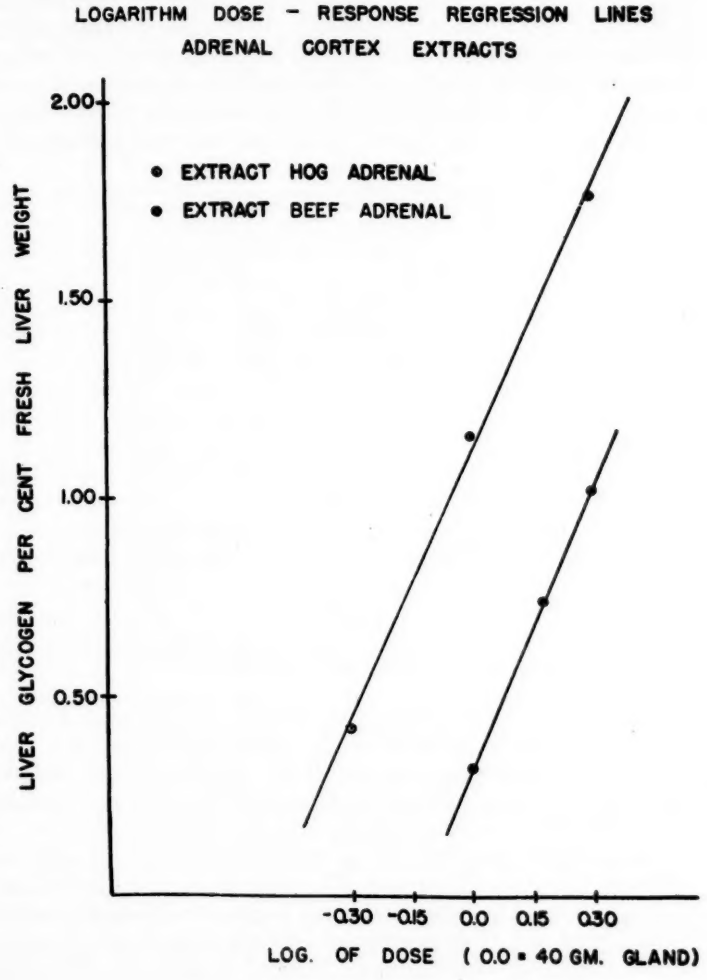


FIG. 4

dro-17-hydroxycorticosterone by both tests is also given in Table 2. Since the slopes for glycogen deposition do not differ significantly accurate evaluations can be made which check remarkably well with those found by the work test and reliably show that for the two

extracts here assayed the one (hog adrenal) was slightly more than twice as active as the other (beef adrenal).

DISCUSSION

The data from a number of other laboratories are relative to the present study. Reinecke and Kendall (1943) listed 11-dehydrocorticosterone, corticosterone and 11-dehydro-17-hydroxycorticosterone for glycogenic activity in the adrenalectomized rat and found ascending potency in the order named, but concluded that there was less than a one-fold difference in activity among these compounds. Olson,

TABLE 2. THE RELATIVE ACTIVITY OF THREE CRYSTALLINE HORMONES OF THE ADRENAL CORTEX AND TWO ADRENAL CORTICAL EXTRACTS COMPARED TO 11-DEHYDRO-17-HYDROXYCORTICOSTERONE

Preparation	Glycogen deposition test			Muscle-work test		
	No. of Animals	Potency ratio %	Error of potency ratio in % at *P = 0.05	No. of animals	Potency ratio %	Error of potency ratio in % at *P = 0.05
11-dehydro-17-hydroxy-corticosterone	50	100	—	135	100	—
Corticosterone	40	52	−13 +14	15	63	−17 +20
11-dehydro-corticosterone	40	48	−12 +13	15	48	−17 +20
17-hydroxy-corticosterone	59	148	−12 +14	15	192	−16 +19
		Gms. of Tissue equivalent to 1 mg. 11-dehydro-17-hydroxycorticosterone			Gms. of Tissue equivalent to 1 mg. 11-dehydro-17-hydroxycorticosterone	
Beef extract	40	216	−14 +17	15	200	−16 +19
Hog extract	39	96	−14 +16	15	83	−16 +19

* Statistical calculation of the error of potency ratio at $p = 0.05$ indicates that 95% of the time these values, within the limits of error, will be obtained in future assays (Burn, 1937).

Thayer and Kopp (1944) assayed the four C-11-oxygenated adrenal steroids for glycogenic potency in the adrenalectomized rat. Corticosterone was slightly less active than 11-dehydrocorticosterone, but the difference was lacking in statistical significance. The compound 11-dehydro-17-hydroxycorticosterone was more potent, and 17-hydroxycorticosterone was the most potent of the four compounds.

Although the order of activity is in agreement with that found in the present study, the latter indicates a greater difference between the potencies of the 17-hydroxylated steroids and those of corticosterone and 11-dehydrocorticosterone. We find 11-dehydro-17-hydroxycorticosterone to be twice as active and 17-hydroxycorticosterone to be three times as active as 11-dehydrocorticosterone, whereas the results of Olson and co-workers indicate a potency compared to 11-dehydrocorticosterone of only 1.16 and 1.3. Statistical analysis of our data by the glycogen test using the rat indicates that the errors of the potency ratios are within ± 20 per cent. The higher order of ac-

tivity for the 17-hydroxylated steroids is also indicated by the muscle work test.

Venning, Kazmin and Bell (1946) studied the effect of various preparations on the level of liver glycogen in the adrenalectomized mouse given glucose. The compound 11-dehydro-17-hydroxycorticosterone was approximately three times as potent as 11-dehydrocorticosterone. Similarly, Dorfman, Shipley, Ross, Schiller and Horwitt (1946) found 11-dehydro-17-hydroxycorticosterone to be four times as potent as 11-dehydrocorticosterone in maintaining the liver glycogen in the adrenalectomized mouse.

The mouse has been found by the above authors and by Eggleston, Johnston and Dobriner (1946) to be most suitable for the detection of small amounts of cortical hormone activity. By comparing the results obtained in mice with those obtained in rats, it is found however that the rat gives much more reliable results. Dorfman has pointed out that the error when groups of 10 mice are used ($p=0.05$) was -59 to $+150$ per cent (Dorfman, Shipley and Ross, 1946), whereas we find the error to be within $\pm$ 20 per cent when rats are used, as described for this study. In this connection, reference by Dorfman to a "pig adrenal cortical extract" supplied by us and used by him in his extensive assay studies deserves comment.

The preparation was found by us to be equivalent in biological activity by the muscle work test to 2 mg. of 11-dehydro-17-hydroxycorticosterone, or approximately 4 mg. of corticosterone per cc. of extract. Actual assays by the glycogen test, applied as described in this report to the extract used by Dorfman, gave a value of 2080 μg. of the standard (11-dehydro-17-hydroxycorticosterone) per cc. The value of 886 μg. reported by Dorfman *et al.* (1946) is not in agreement with the results obtained by us. A larger assay error by the mouse than by the rat liver-glycogen method may partially explain this. Another contributing factor may be that Dorfman obtained different potency ratios by the mouse liver-glycogen method for the C-11-oxygenated steroids than we obtained with the rat method. Dehydrocorticosterone was found by us, for example, to be 48 per cent as active as 11-dehydro-17-hydroxycorticosterone, whereas Dorfman found it to be only 25 per cent as active.

Although the ratios of activity among the C-11-oxygenated steroids have been reported somewhat differently from the various laboratories using the liver-glycogen depostition method, there is good agreement that the compounds hydroxylated at C-17 are the most potent. This conclusion is also in accord with the results of the muscle work test.

There may be some question as to whether the same biologic response is measured by the work test and the glycogen deposition test. The results are quite definite, however, that the relative potencies of the pure C-11-oxygenated steroids and the beef and hog ad-

renal extracts are the same by either of these tests. The hog adrenal extract was found by both methods to be twice as potent as the beef on the basis of amount of gland extracted. Since the regression lines for the two extracts are not significantly different from that of the standard 11-dehydro-17-hydroxycorticosterone by the glycogen method, it appears that this would constitute a satisfactory assay procedure for adrenal cortical preparations and that the unit of activity of 0.2 mg. of 11-dehydro-17-hydroxycorticosterone previously defined for the muscle work test is also suitable for assays by glycogen deposition.

There is clinical evidence for validating bio-assay methods for carbohydrate functions. Studies by MacBryde and De La Balze (1944), Forsham, Thorn and Bergner (1946) and Sprague and Kepler (1946) support conclusions that hog adrenal extract is more potent per unit of gland than beef adrenal extract, and that 17-hydroxy-11-dehydrocorticosterone is highly active in its effect on carbohydrate metabolism. These clinical studies have also shown that 11-dehydrocorticosterone is only weakly active in patients with Addison's Disease. This lower degree of activity is also indicated by our bio-assay data.

We feel, however, that it is important to emphasize that if carbohydrate function tests are used exclusively important fractions of the extract may be overlooked. It would seem desirable to continue the use of the survival-growth test, which is responsive to the composite activity of adrenal cortex extracts, since the prolongation of survival of adrenalectomized animals and of patients having adrenal cortical insufficiency is an important objective to be obtained in the preparation of these extracts.

SUMMARY

Comparative results by liver-glycogen deposition and muscle work performance bio-assay methods have been tabulated for four crystalline hormones of the adrenal cortex and two adrenal cortical extracts.

The order of activity for the preparations studied was the same by both methods. 17-Hydroxycorticosterone was found to be more active than 17-hydroxy-11-dehydrocorticosterone. Corticosterone and 11-dehydrocorticosterone were found to be considerably less active than their 17-hydroxy derivatives. Hog adrenal cortical extract was found to be twice as potent per unit weight of adrenal glands as a corresponding beef adrenal extract.

The results by the glycogen test, employing adrenalectomized rats, were found to be satisfactorily reliable and indicate that this method is suitable for bio-assay purposes.

Since the slopes of the regression lines for glycogen deposition response for all of the C-11-oxygenated steroids and adrenal cortical

extracts are not significantly different, any one of the steroids can serve as a reference standard for bio-assay by this method.

ACKNOWLEDGMENTS

The authors are grateful to Dr. R. I. Dorfman, who, by a different method of statistical analysis of the liver-glycogen response data, obtained the same conclusions as our own regarding the reproducibility of results by this assay procedure.

We are greatly indebted to Dr. D. J. Ingle for his helpful criticism of this work.

REFERENCES

Bergman, H. C., and David Klein: *Endocrinology* 33: 174. 1943.
Burn, J. H.: Biological Standardization, Oxford University Press, London, 1937.
Cartland, G. F., and M. H. Kuizenga: *Am. J. Physiol.* 117: 678. 1936.
Dorfman, R. I., R. A. Shipley and E. Ross: *Endocrinology* 38: 178. 1946.
Dorfman, R. I., R. A. Shipley, E. Ross, S. Schiller and B. N. Horwitt: *Endocrinology* 38: 189. 1946.
Eggleston, N. M., B. J. Johnston and K. Dobriner: *Endocrinology* 38: 197. 1946.
Fisher, R. A.: Statistical Methods for Research Workers, Oliver and Boyd, Edinburgh, 1932.
Forsham, P. H., G. W. Thorn and G. E. Bergner: *Am. J. Med.* 1: 105. 1946.
Good, C. A., H. Kramer and M. Somogyi: *J. Biol. Chem.* 100: 485. 1933.
Ingle, D. J.: *Endocrinology* 34: 191. 1944.
Ingle, D. J., and J. G. Griffith: Chapter 16. The Rat in Laboratory Investigation. J. B. Lippincott Co., Philadelphia. 1942.
Ingle, D. J., and M. H. Kuizenga: *Endocrinology* 36: 218. 1945.
Irwin, J. O.: Supplement to *J. of Roy. Stat. Soc.* 4: 1. 1937.
Kuizenga, M. H., J. W. Nelson and D. J. Ingle: *Am. J. Physiol.* 139: 499. 1943.
Kuizenga, M. H., J. W. Nelson, S. C. Lyster and D. J. Ingle: *J. Biol. Chem.* 160: 15. 1945.
Kuizenga, M. H., A. N. Wick, D. J. Ingle, J. W. Nelson and G. F. Cartland: *J. Biol. Chem.* 147: 561. 1943.
MacBryde, C. M., and F. A. De La Balze: *J. Clin. Endocrinol.* 4: 287. 1944.
Olson, R. E., F. A. Jacobs, D. Richert, S. A. Thayer, L. J. Kopp and Nelson J. Wade: *Endocrinology* 35: 430. 1944.
Olson, R. E., S. A. Thayer and L. J. Kopp: *Endocrinology* 35: 464. 1944.
Reinecke, R. M., and E. C. Kendall: *Endocrinology* 31: 573. 1942.
Reinecke, R. M., and E. C. Kendall: *Endocrinology* 32: 505. 1943.
Shaffer, P. A., and M. Somogyi: *J. Biol. Chem.* 100: 695. 1933.
Sprague, R. G., and E. J. Kepler: Personal Communication.
Venning, E. H., V. E. Kazmin and J. C. Bell: *Endocrinology* 38: 79. 1946.

DETERMINATION OF THE RELATIVE ACTIVITIES OF ANTITHYROID COMPOUNDS IN MAN USING RADIOACTIVE IODINE[1]

MALCOLM M. STANLEY,[2] M.D. AND E. B. ASTWOOD, M.D.
From the Joseph H. Pratt Diagnostic Hospital and the Department of Medicine, Tufts Medical School
BOSTON, MASSACHUSETTS

HERETOFORE, in the study of compounds which inhibit the function of the thyroid gland, methods for the evaluation of potency have depended upon the goitrogenic action of the drugs in animals such as the immature rat (Astwood, Bissell, and Hughes, 1945) or chick (VanderLaan and Bissell, 1946). The primary action of such substances is to prevent the synthesis of thyroxin; under their influence the stored hormone continues to be secreted and consequently the iodine content of the thyroid gland decreases rapidly. The eventual lowering of the thyroid hormone level in the blood stimulates increased secretion of thyrotropin from the pituitary, which causes enlargement of the thyroid. Thus, the depletion of iodine from the thyroid and its increase in weight are indirect measures of inhibition of thyroxin synthesis, and hence of the antithyroid potency of the compound.

The principal disadvantage of a method of assay employing animals is the uncertainty that the results obtained will be applicable to man. The limitations of the rat test in determining the antithyroid potency of compounds to be used in man may be seen by comparing the effectiveness of such compounds as thiourea, thiouracil, methylthiouracil, thiobarbital, and propylthiouracil. In the rat these compounds have relative activities of 0.12, 1.00, 1.00, 1.7, and 11.0 respectively (Astwood, Bissell and Hughes, 1945), while in man their relative activities are quite different. According to clinical trial propylthiouracil appears to be only slightly more active than thiouracil, thiobarbital is highly active, while thiourea and methylthiouracil are more active in man than the animal assays would indicate.

Ideally, drugs which are to be used for the treatment of thyrotoxicosis should be assayed by a method which determines their effect on

Received for publication May 20, 1947.
[1] Aided in part by grants from the Committee on Endocrinology of the National Research Council and the American Cyanamid Company, Stanford, Connecticut.
[2] Fellow of the American Cancer Society recommended by the Committee on Growth of the National Research Council.

subjects with this disease. An analogous method, for example, is that which employs patients with pernicious anemia for the standardization of liver extract. However, the assay of antithyroid compounds on patients with hyperthyroidism by evaluating the response to treatment is most difficult and time consuming. Marked variation in the rate of response to full doses of an antithyroid agent is a common clinical observation; there also appears to be a considerable individual variation in the minimal effective dose in different patients. As the rate of response to therapy may be conditioned by the quantity of hormone stored in the thyroid gland, it is most difficult to say whether a slow response is due to this or to a suboptimal dose. Also, since the number of available thyrotoxic patients is limited, it would be of more practical value to be able to test compounds in the normal human subject.

The possibility of using radioactive iodine for this purpose was suggested by a consideration of the mechanism of action of antithyroid compounds. Recent studies have clearly differentiated two separate and successive steps in the iodine concentrating mechanism of the thyroid gland (VanderLaan and VanderLaan, 1947). The first step involves the concentration of iodide ion by thyroid tissue; normally this accounts for only a small fraction of the total iodine accumulated by the gland and it is this phase of iodine metabolism which is specifically inhibited by thiocyanate ion. The second mechanism which accounts for the major quantity of iodine accumulated in the normal gland involves the conversion of the entrapped iodide ion to organically bound iodine; it is this step which is inhibited by antithyroid compounds of the thiouracil type. Consequently, the administration of an antithyroid compound greatly decreases the accumulation of iodine by the thyroid gland of normal individuals.

Use has been made of this effect by McGinty and Rawson; they assayed various antithyroid compounds in rats by administering the compound shortly before the injection of radioactive iodine. The inhibition of iodine accumulation in the thyroid gland as measured by radioactivity counts on the dissected and prepared thyroid glands has served as a test of the effectiveness of the antithyroid agent. The demonstration by Hamilton and Soley (1939, 1940) and by Hertz, Roberts, and Salter (1942) that the rate and extent of accumulation of radioactive iodine by the human thyroid gland could readily be determined with an externally placed Geiger counter suggested that this method might be applicable to the assay of antithyroid compounds.

It is the purpose of this paper to describe a method of assay of antithyroid compounds which depends upon their property of inhibiting the uptake by the thyroid of radioactive iodine. The results of assay of 32 compounds are also described.

METHODS

The subjects were normal people of both sexes and included physicians, nurses, laboratory technicians, and other hospital workers who volunteered. The isotope, I^{131} with a half-life of 8.0 days, obtained from the Clinton Laboratories, Oak Ridge, Tennessee, in the form of a weakly alkaline solution, was used. In all instances a "tracer" dose of 0.1 millicurie, without added carrier, was diluted with saline and given by mouth, followed by a little water to wash the material from the vessel, mouth, and esophagus. The iodine was usually administered during the hour following consumption of a normal breakfast, except in a few instances, in which the subjects were fasting.

The measurements of accumulation of radioactive material by the thyroid were made with a "Victoreen" Geiger-Muller counting tube with a 3 cm. end window enclosed with the preamplification circuit in a metal cylinder 20 cm. long and 3.5 cm. in diameter. The tube was surrounded by a lead shield 1 cm. thick; the mica window of the tube was covered by a second sheet of mica cemented to the housing to exclude moisture and by a cap of brass 0.8 mm. thick (0.67 gm./sq. cm.). The purpose of the latter was to insure exclusion of beta radiation; any contamination of the skin surface with radioactive iodine, as through its excretion in the sweat, would give rise to large errors if this precaution were not taken. This brass filter reduced the gamma radiation by approximately 15 per cent. The recording apparatus was an "Autoscaler" made by Tracerlab Inc., of Boston. The time required for any predetermined number of counts in multiples of two up to 4096 is registered; from this the rate of counting, expressed in terms of counts per second, is readily calculated. During the actual counting the subjects sat erect in a straight chair; the counter was pressed against the midline of the neck near the isthmus of the thyroid in such a way that the rim of the cap was resting on the sternal notch. Usually, four determinations requiring from 20 to 40 seconds each were averaged at each counting period. Counts were made at 20- to 30-minute intervals during the first few hours when uptake was most rapid, every hour thereafter for the rest of the day, and usually at 24 hours following administration of the iodine; in many instances, the levels were followed over a period of weeks by counts every few days. Determinations of the background count and checks on the accuracy of the apparatus with a uranium standard were made at appropriate intervals. Correction was made for the physical decay of the iodine.

PATTERN OF ACCUMULATION OF IODINE BY THE THYROID GLANDS IN NORMAL CONTROL SUBJECTS

During the period of from 30 to 60 minutes following ingestion of the iodine, while absorption from the gastrointestinal tract was incomplete, there was generally a slow increase in accumulation of radioactivity which was probably influenced somewhat by the amount and character of the stomach contents; this segment of the pattern was absent when the material was injected intravenously. Soon thereafter as absorption neared completion and the concentration in the body fluids approached a maximum, there was a rapidly increasing rate of uptake in the thyroid. After this, due to the steadily decreas-

ing concentration of radioactive material in the body fluid reservoir from excretion in the urine and accumulation by the thyroid, or due to the dilution of the radioactive iodine by endogenous and exogenous ordinary iodide, there was a gradual and progressive slowing of the rate of uptake until a plateau was attained at 24 to 48 hours (Figure 1a). During the next several weeks, the concentration usually remained remarkably constant. The curve of accumulation resembled, therefore, a greatly distorted "S."

ACCUMULATION GRADIENT

Because of the variations which were encountered in the rate of iodine accumulation in different subjects, a reliable method of prediction of the pattern of uptake from observations made during the first few hours was a necessity if a change following the application of a modifying agent were to assume significance. A useful approximation utilized the fact that the major portions of the "S" shaped curve comprised a parabola (Figure 1a). Thus, if this curve were plotted so that the abscissa was the square root of the elapsed time, the values during the first eight hours or so could usually be fitted to a straight line (Figure 1b). During the first thirty minutes of the uptake period, the points were usually above this line; after eight hours, they tended to fall below it. In general, it seemed that the more rapid the uptake the shorter the time over which this relationship held. In individuals who had a very rapid accumulation of iodine, the pattern of uptake could be fitted to a straight line only over a brief period of time by this method; conversely, in those subjects with a very low rate of accumulation, the straight line relationship obtained for as long as 24 hours. Although not a strict mathematical expression of the accumulation pattern, this method was useful in the assay of the antithyroid compounds and had the advantage of simplicity.

The slope of this straight line has provided a convenient means of calculating an arbitrary numerical expression of the rate of uptake, which has been termed the "accumulation gradient." The gradient can be computed from any two points on this line. It is obtained by dividing the increase in radioactivity during a given period, expressed as counts per second (per 0.1 millicurie dose), by the number resulting from subtracting the square root of the time in minutes at the beginning of the period from the square root of the time in minutes at the end of the period. For example, in figure 1b (IV), if the number of c/s increased from 31 to 139 in the interval between 36 minutes and 256 minutes after the tracer dose was ingested, the accumulation gradient was calculated as follows:

$$G. = \frac{139 - 31}{\sqrt{256} - \sqrt{36}} = \frac{108}{16 - 6} = 10.8.$$

In the first 60 normal subjects tested, this has varied between the

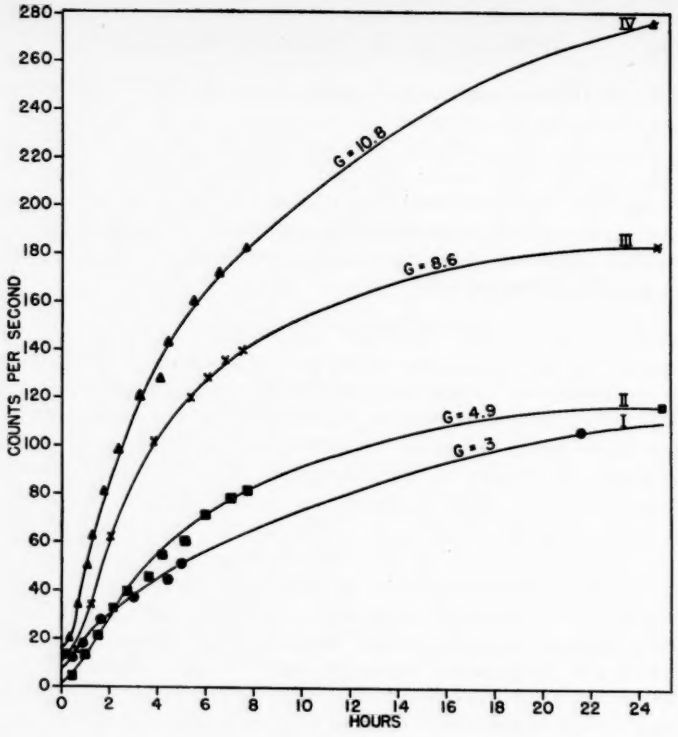

COUNTS PER SECOND
HOURS
G = 10.8
G = 8.6
G = 4.9
G = 3
IV
III
II
I

FIG. 1a

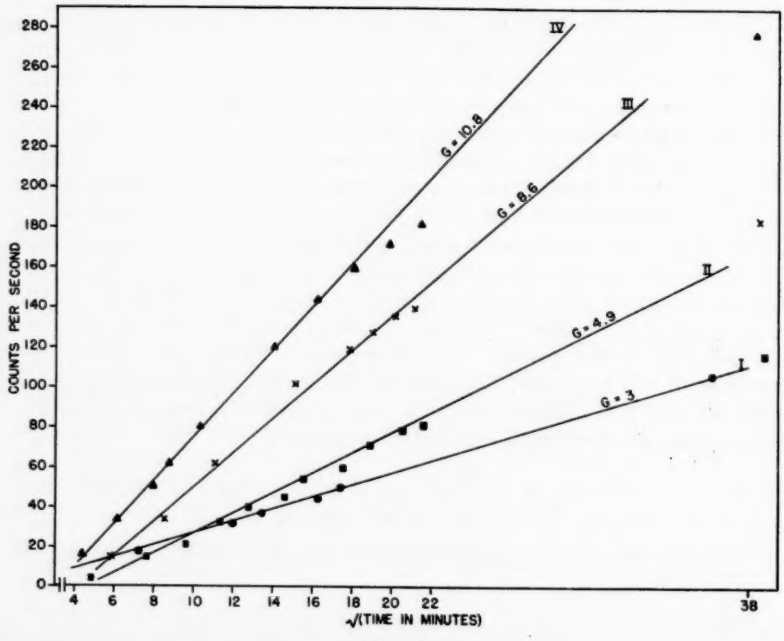

COUNTS PER SECOND
√(TIME IN MINUTES)
G = 10.8
G = 8.6
G = 4.9
G = 3
IV
III
II
I

FIG. 1b

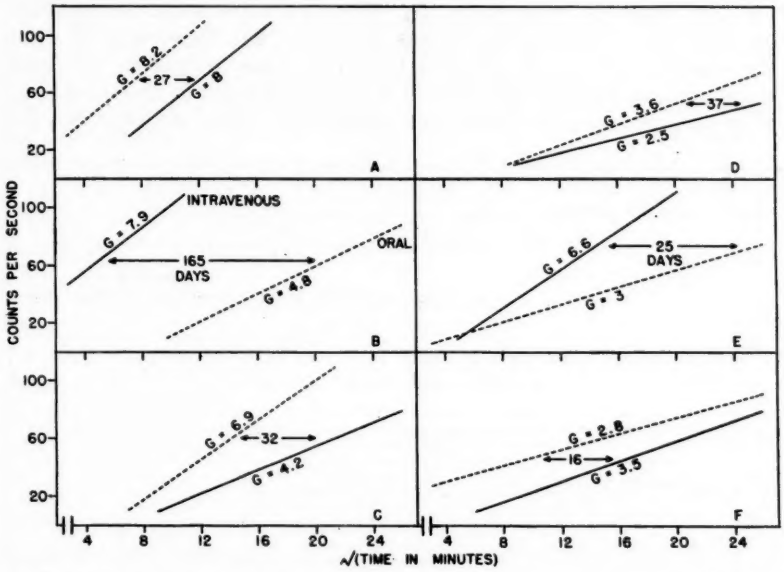


FIG. 2. The rates of uptake of radioactive iodine in six normal subjects tested twice are plotted as in figure 1b. The solid lines indicate the first experiment, the dotted lines the second; the figures between the two curves reveal the interval in days between the two determinations. In b the first study was done with the radioactive iodine injected intravenously; in b, the second, and in all the others shown the material was administered orally. In some subjects (a, d, f), there was close agreement between the results from the first and second determinations. In the others, the rates of accumulation varied somewhat; in subjects b and e, the first tests showed the higher gradient, while in subject c the second determination revealed the more rapid uptake.

limits of 1.4 and 36.4 with a mean of 9.4 (Astwood and Stanley, 1947).

These very considerable variations in accumulation gradient occurred among individuals who were clinically well and who, therefore, must be considered as normal. The experience thus far gained is not broad enough to show whether these variations are due to differences in the iodine intake in the diet, or whether they represent differences in the rate of thyroid hormone synthesis, either inherent in the gland itself, or as the result of changes in metabolic demands

FIG. 1. The accumulation of radioactive iodine by the thyroid in four normal subjects with different rates of uptake. In (a) the counts per second, as detected by an externally placed Geiger counter, are plotted against elapsed time in hours. The resulting curves resemble greatly distorted "S's" with the major portions of each comprising parabolas. In (b) the same data are plotted so that the abscissa is the square root of the elapsed time in minutes. From the slopes of the straight lines obtained, arbitrary numerical expressions of the rates of uptake, G ("accumulation gradient"), are derived as explained in the text.

for the hormone occasioned by season, temperature, occupation, activity, etc. Furthermore, it was observed that there may be some disparity in pattern of accumulation from time to time in the same individual, although frequently it was quite similar in those subjects who were tested more than once (Figure 2). These observations are

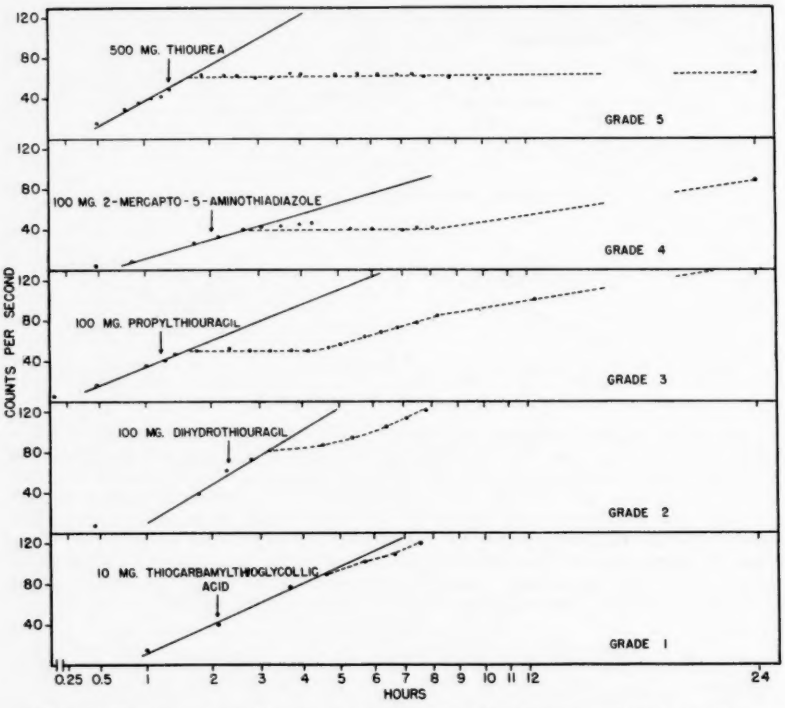


FIG. 3. These five experiments were selected as typical examples of the five arbitrary degrees of inhibition which were used to designate the response to an antithyroid compound. 500 mg. of thiourea completely prevented the accumulation of iodine in the thyroid gland for twenty-four hours; 100 mg. of 2-mercapto-5-amino-1,3,4-thiadiazole was completely inhibitory for more than four but less than twenty-four hours; 100 mg. of propylthiouracil completely inhibited uptake for less than four hours; 100 mg. of dihydrothiouracil exhibited a partial effect; the response to 10 mg. of thiocarbamylthioglycollic acid was questionable or only slight.

in harmony with the concept that the level of thyroxin production and secretion is a constantly changing value precisely adjusted to varying metabolic requirements.

ASSAY OF ANTITHYROID COMPOUNDS

In the testing of antithyroid agents it was found convenient to plot the radioactivity measurements directly against the square root of the elapsed time (in minutes). Following the administration of the

radioactive iodine, measurements were made and the values plotted until it was clear that sufficient time had elapsed so that the straight line relationship had become established. This usually required from one to two hours. The dose of antithyroid agent was then administered by mouth and the subsequent course of the iodine accumulation was determined and plotted on the graph. The degree to which the predicted accumulation was modified was then graded according to an arbitrary system wherein various scores of 0 to 5 were assigned on the bases of the completeness and the duration of inhibition. The various degrees of inhibition were classified as follows:

0 = No effect.
1 = Slight or questionable inhibition.
2 = Definite but incomplete inhibition.
3 = Complete inhibition lasting less than 4 hours.
4 = Complete inhibition for more than 4 but less than 24 hours.
5 = Complete inhibition for more than 24 hours.

Typical examples of these different degrees of inhibition are shown in Figure 3.

Effects of Individual Variations in the Test Subjects. Since this method was to be used as a means of evaluating the potency of antithyroid compounds, studies were undertaken to determine its reliability and constancy in subjects whose rates of accumulation varied widely. Five subjects, with accumulation gradients ranging from 5.8 to 14.2, were given 100 mg. each of propylthiouracil; in all of these, despite marked differences in initial rates of uptake, the effects were quantitatively so similar that each was assigned exactly the same rating. A number of similar examples of constant response to the same dose of drugs among different subjects may be seen from Table I. In very few instances did the response to a given dose vary more than one grade. Although there is certain to be some difference in response among the members of any group, this variation is not enough to preclude the random choice of normal individuals as test subjects.

Effect of Variation of the Dosage of a Compound. In preliminary tests, amounts of drugs which might be expected to produce a pronounced effect were given. In later studies, active compounds were given in progressively smaller doses so that the minimum dose which produced a definite response was determined. With most compounds there was a nice gradation of effect, with the degree of response proportional to the dose. Figure 4 shows the effect of varying amounts of mercaptoimidazole given in doses of from 5 to 25 mg.

Toxic Effects. All of the drugs tested had previously been administered to animals, so that an estimate of their toxicity had been obtained before they were given to human subjects. However, the toxicity of the majority of the drugs assayed had not been evaluated by clinical trial, and, therefore, only one dose of any one substance

was administered to any subject. With this simple precaution, it was anticipated that no toxic effects would be experienced by the test subjects, since such untoward reactions are almost invariably the result of sensitization from repeated ingestion of a drug. A transient macular rash about the face occurred in one subject several hours

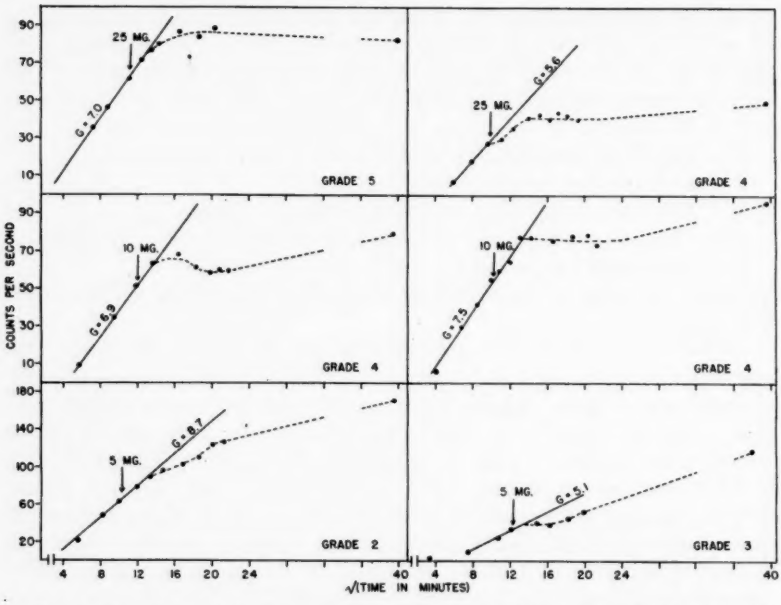


FIG. 4. The effects of three doses, 5, 10, and 25 mg., of 2-mercapto-imidazole in six normal subjects. 25 mg. caused complete inhibition for twenty-four hours in one subject and for less than twenty-four hours in the other. Complete inhibition for more than four but less than twenty-four hours was observed in both subjects with 10 mg. while 5 mg. produced partial inhibition in one subject and complete but transient inhibition in the other.

after the administration of 500 mg. of thiourea. This was the only undoubted instance of an undesirable effect encountered in the whole series; in two other subjects, similar rashes occurred, but the time relationships (four days after the test) were such that other etiologic factors could not be excluded.

RELATIVE ACTIVITIES OF THE COMPOUNDS EMPLOYED

In Table I, the results of testing 32 compounds in 90 subjects by this method are summarized. For purposes of comparison, the estimated activities of these same compounds when assayed by the rat method are also listed. It is evident from Table I that there is a marked difference in the results obtained by the rat method of assay and the present procedure using human test subjects.

Thiouracil and Its Derivatives. When assayed by the former method, the 6-n-propyl derivative was the most potent of the substituted thiouracils (11 times that of thiouracil); also more effective than thiouracil were 6-benzyl (10 times); 6-tertiary butyl (9 times); 6-isopropyl (9 times); 6-ethyl (8 times) in order of decreasing strength. The effectiveness of 6-methylthiouracil was about equal to that of thiouracil itself. 6-aminothiouracil was quite impotent in the rat.

When assayed in normal human beings, 6-aminothiouracil was quite inert in the dosage used. 6-tertiary butylthiouracil was barely active in a dose of 100 mg. in man, but it was nine times as active as thiouracil when tested in the rat. Of the other ten 6-substituted thiouracils tested in man, the methyl derivative appeared to be the most potent; it was twice as active as thiouracil itself, and four times as effective as the weaker compounds, such as 5, 6-dihydro- and 6-n-hexylthiouracil. The 6-ethyl and 6-cyclopropyl derivatives occupy an intermediate position; they apparently were about equal to thiouracil in their antithyroid effects in man. 6-n-propylthiouracil and 6-benzylthiouracil were somewhat less active than thiouracil though the rat tests showed them to be eleven and ten times as active respectively.

In general, it would seem that the various 6-substituted thiouracils are more uniform in their antithyroid activity in man than in the rat. Addition of an alkyl group in the 6-position, from the small number of compounds tested, does not result in a marked increase in potency in human beings as it does in some instances when tested in the rat.

Though the molecular structure of tetrahydro-2-thiopyrimidone differs considerably from thiouracil, these two substances possess similar degrees of antithyroid activity. This result, again, is at variance with the assay by the rat method by which it was found that tetrahydro-2-thiopyrimidone was approximately one-eighth as potent as thiouracil.

Thiobarbital (5, 5-diethylthiobarbituric acid). This was found to be about twice as potent as thiouracil in normal human subjects by this method. From rat tests, figures of like magnitude (1.2 and 1.7) were also obtained. The efficacy of thiobarbital in the treatment of thyrotoxicosis has been demonstrated by clinical trial. From the small size of the dose (100 mg. daily, or less) which is usually required it seemed to be several times more potent than thiouracil. This seeming or actual difference between results on clinical cases and those by the present method may be due to other factors to be discussed subsequently.

Thioureas. Of the three thioureas studied, the parent substance thiourea itself and 1-isopropylthiourea seemed to exert about as much antithyroid action as thiouracil. 1, 3 diisopropylthiourea was somewhat less active in man. In rats, thiourea was only one-eighth to one-

TABLE I. THE DEGREES OF RESPONSE TO VARIOUS DOSES OF 32 COMPOUNDS WHEN ADMINISTERED TO HUMAN SUBJECTS. THE VALUES 0 TO 5[1] SIGNIFY INCREASING GRADES OF INHIBITION OF RADIOACTIVE IODINE ACCUMULATION IN THE THYROID GLAND

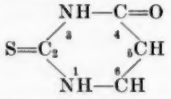
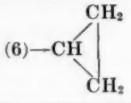
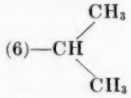
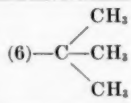
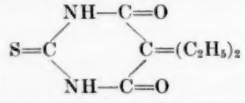
No.	Compound	Formulae and substituents
1	Thiouracil	
2	Dihydrothiouracil	(5)—H, (6)—H
3	Tetrahydro-2-thiopyrimidone	(4)—H_2, (5)—H, (6)—H
4	6-Methylthiouracil	(6)—CH_3
5	6-Ethylthiouracil	(6)—CH_2CH_3
6	6-Cyclopropylthiouracil	
7	6-Isopropylthiouracil	
8	6-*n*-Propylthiouracil	(6)—$CH_2 \cdot CH_2 \cdot CH_3$
9	6-*Tert.* Butylthiouracil	
10	6-*n*-Butylthiouracil	(6)—$CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_3$
11	6-*n*-Hexylthiouracil	(6)—$CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_3$
12	6-Benzylthiouracil	
13	6-Aminothiouracil	(6)—NH_2
14	Thiobarbital	
15	Thiourea	$H_2N \cdot C(S) \cdot NH_2$
16	1-Isopropylthiourea	$H_2N \cdot C(S) \cdot NH \cdot CH(CH_3)_2$
17	1,3-Diisopropylthiourea	$(CH_3)_2CH \cdot NH \cdot C(S)NH \cdot CH(CH_3)_2$
18	Thiocarbamylthioglycollic acid	$H_2N \cdot C(S) \cdot S \cdot CH_2COOH$

TABLE I—*Continued*

No.	Degrees of response[1]							Estimated activity (Thiouracil = 1.0)		
	Dose in milligrams							Rat[2]		Man
	5	10	25	50	100	200	500			
1			1	2, 3	4, 4, 3	4		1.00	1.00	1.0
2					2				0.08	0.5
3		0, 0			4				0.13	1
4			2	4, 3	4			1.04	1	2
5				2	4				8	1
6				2	4				8[3]	1
7				2					9	1
8				2, 1, 1	3, 3, 3 3, 3		4		11	0.75
9					0, 2				9	0.25
10				2	3				3	0.75
11					2				0.18	0.5
12				2	3	4			10	0.75
13					0				<0.001	<0.1
14		1	2, 2		4			1.23	1.7	2
15				2, 2	3		5	0.09	0.12	1
16					3				0.4	1
17					1				0.3	0.25
18		1, 2			4				0.4	2.5

TABLE I—*Continued*

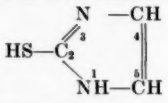
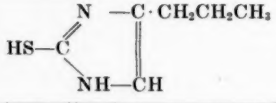
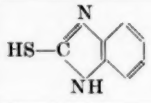
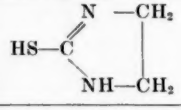
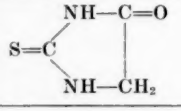
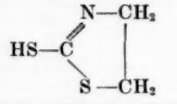
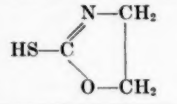
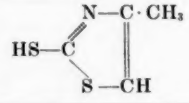
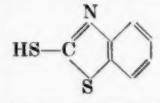
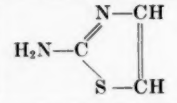
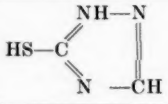
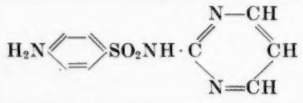
No.	Compound	Formulae and substituents
19	2-Mercaptoimidazole	
20	2-Mercapto-4-propylimidazole	
21	2-Mercaptobenzimidazole	
22	2-Mercaptoimidazoline	
23	2-Thiohydantoin	
24	2-Mercaptothiazoline	
25	2-Mercaptooxazoline	
26	2-Mercapto-4-methylthiazole	
27	2-Mercaptobenzothiazole	
28	2-Aminothiazole	

TABLE I—*Continued*

No.	Degrees of response[1]							Estimated activity (Thiouracil = 1.0)		
	Dose in milligrams							Rat[2]		Man
	5	10	25	50	100	200	500			
19	2, 2, 2 1, 3, 3	4, 4 2, 4, 3	4, 5		5				1.5[3] 2.0	10
20					3				1[3]	0.75
21			3		4			1.16	0.5	2.5
22					2			0.63	0.4	0.5
23		2, 1, 2			4				0.4	2.5
24			3, 2		4			1.31	1.3	2.5
25					3				0.2	0.75
26			2					0.095	<0.1	1
27					1			0.02	0.005	0.1
28			3		5			0.06[4]	0.15[3] 0.05	2.5

TABLE I—*Continued*

No.	Compound	Formulae and substituents
29	3-Mercapto-1,2,4-triazole	
30	2-Mercapto-5-amino-1,3,4-thiadizaole	
31	Sulfadiazine	
32	Potassium thiocyanate	KSCN

eleventh as effective as thiouracil; the addition of one or two propyl groups increased the potency of the compounds three to four times, but they still remained relatively inert. Thiocarbamylthioglycollic acid, a straight chain compound with an $= N - \overset{\overset{S}{\|}}{C} - S -$ grouping was about 2.5 times as effective as thiouracil in man. This substance was the only one of a group of related compounds with appreciable activity in the rat (0.4 times thiouracil).

Imidazoles and Imidazolines. These compounds, 5-membered heterocyclic ring structures containing thioureylene groupings, varied greatly in their antithyroid activity. 2-mercaptoimidazole itself was the most potent of all the compounds tested in man. In doses as low as 5 mg. this material exerted appreciable antithyroid effects in most instances (Figure 4,) while 10 mg. appeared to inhibit hormone synthesis as effectively as 100 mg. of thiouracil. Mercaptoimidazole was only 1.5 times as effective as thiouracil in the rat. No long-term animal toxicity studies have been carried out with this substance, but previous short-term animal experiments (Astwood, Bissell, and Hughes, 1945) revealed no unusual toxic effects.

Other related compounds which were more powerful than thiouracil are mercaptobenzimidazole (2.5 times) and 2-thiohydantoin (2.5 times). Mercapto-4-propylimidazole (.75) and mercaptoimidazoline (ethylenethiourea) (.5 times) were less active than thiouracil.

Thiazoles, Thiazolines, and Oxazolines. This group of compounds differs from the preceding in that one of the N atoms is replaced by

TABLE I—*Continued*

No.	Degrees of response[1]							Estimated activity (Thiouracil = 1.0)		
	Dose in milligrams							Rat[2]		Man
	5	10	25	50	100	200	500			
29			2		5				0.3	2
30			2	3	4			1.56	1.2	2
31							0		0.1	<0.05
32							2[5] 2, 2		—[6]	—

[1] The degrees of response were classified as follows: 0 = no effect; 1 = slight or partial effect; 2 = distinct but incomplete inhibition; 3 = complete inhibition for less than 4 hours; 4 = complete inhibition for more than 4 but less than 24 hours; 5 = complete inhibition for 24 hours.
[2] The values in the first column are from McGinty and Bywater; Bywater, McGinty and Jenesel (1945) and those in the second from Astwood, Bissell and Hughes (1945).
[3] Unpublished data.
[4] Personal communication from Dr. D. A. McGinty.
[5] Dose of 1.0 gm. of potassium thiocyanate.
[6] Thiocyanate was found to be inactive in rats unless the dietary iodine is low.

S or O. The change in potency thus produced is not predictable or constant among the few members tested. Mercaptobenzimidazole is at least ten times as potent as mercaptobenzothiazole while mercaptoimidazoline has less than one-fifth the activity of mercaptothiazoline and is about equal to mercaptooxazoline.

2-aminothiazole was the most effective member of this group, corroborating the clinical studies which have demonstrated its efficacy in the treatment of thyrotoxicosis. It is the only compound tested in which the sulfur atom cannot exist in a free thio form. 4-methyl-2-mercaptothiazole, the closest thiazole analog of mercaptoimidazole available to us, seemed only slightly more potent than thiouracil; this is in sharp contrast, however, to its low antithyroid activity in the rat.

Other Heterocyclic Compounds. 3-mercapto-1,2,4-triazole was the only cyclic compound assayed in this study which contained 3 N atoms in the ring structure; it was the only triazole possessing significant potency in the rat (0.3 that of thiouracil). In man, it was about twice as effective as thiouracil. 2-mercapto-5-amino-1,3,4-thiadiazole was slightly more active than thiouracil in the rat (1.2 or 1.56

times) and about twice as potent in man, figures which agree surprisingly well in view of the conflicting results from most of the other compounds tested by the two methods.

Sulfadiazine, the only member of the aminobenzene group of antithyroid compounds tested, seemed to be inert in the human being. This compound is slightly active in the rat but almost inert in the chick. There have been no reports of a goitrogenic action of any of the sulfonamides though they have been administered over long periods of time for prophylaxis of streptococcal infections; it is therefore possible that the members of the aminobenzene series of compounds are of relatively little importance as antithyroid agents in man.

DISCUSSION

The most obvious limitation to the method used involves its failure to reveal possible cumulative effects of the various compounds. It is conceivable that certain substances might so accumulate in the tissues that a large dose, or repeated smaller doses would have to be given before an effective concentration in the body fluids would be achieved. A compound which behaved in this way might then exert an action for a long period of time, and effective concentrations might be maintained by quite small daily doses once the body store had been built up. As antithyroid therapy involves continued treatment over many months, cumulative effects assume major importance and are not readily studied in the above method. Some indication of possible cumulative effects is provided, however, by relating different doses of a given substance to effectiveness and to duration of action. For example, thiourea might be expected to be more cumulative than propylthiouracil for in a dose of 100 mg. propylthiouracil was somewhat the more active, while in the larger dose of 500 mg. thiourea had the more prolonged effect. Short-acting compounds, such as propylthiouracil and other 6-substituted thiouracils, might reasonably be expected to have little cumulative effect, while the longer acting ones, like thiobarbital, might be slowly eliminated. These considerations suggest caution in directly translating the assay values into dosages for prolonged and continued antithyroid therapy.

The actual merits of this method of assay could be better evaluated if there were available exact data on the relative effectiveness of the several antithyroid compounds which have been used in the treatment of hyperthyroidism. Unfortunately the evaluation of the effectiveness of various compounds in therapy is so difficult that exact data are unavailable. Thiourea, for example, has been used in doses varying from 0.4 (Hercus, 1946) to 9.0 grams (Poate and Spencer, 1946) daily with similar effect and no data on the minimal effective dose have been reported. Thiouracil, likewise, is known to be uniformly effective in hyperthyroidism when given in a dose of 0.4 to 0.6 grams

daily, but few data exist on its effectiveness is smaller doses. Propylthiouracil has been found effective in as small a dose as 50 or 75 mg., but most observers (Reveno, 1947; Bartels, 1947) now agree that 150 to 300 mg. are needed in some cases. Consequently, despite extensive clinical trial, few data exist permitting a comparison of the relative activities of thiourea, thiouracil, and propylthiouracil. The finding that according to this test propylthiouracil is no more active than thiouracil or thiourea may not, therefore, be out of keeping with the observed effects of these various agents in therapy. Several investigators have observed methylthiouracil to be more active than thiouracil (Leys, 1945; Meulengracht and Schmitt, 1945; Wilson, 1946); the value obtained by the radioactive iodine method would be consistent with these observations.

The assay value obtained for thiobarbital (two times thiouracil) is considerably lower than previous impressions on its clinical effectiveness would have indicated. Limited trial had shown it to be several times more active than thiouracil; one estimate being ten times (Bartels, 1945), another more than two times (Astwood, 1945). Bartels has further shown that a single daily dose was as effective as multiple doses. The iodine test would indicate that, while the compound in small doses is only twice as active as thiouracil, larger doses exerted a prolonged effect. If this observation were to indicate a tendency to a cumulative effect, then the assay values and the clinical effectiveness would be better reconciled.

Aminothiazole was considered by Perrault and Bovet (1946) to be more active than thiouracil; the present assay value of 2.5 would seem to confirm this. However, in the case of 2-mercaptobenzimidazole (*o*-phenylenethiourea) the iodine test showed an activity of two times thiouracil while Williams (1947) did not find a greater effectiveness in human hyperthyroidism.

On the whole, however, the comparison of the values obtained above with those to be deduced from therapeutic trials are in fair agreement. In view of these considerations, the high antithyroid activity found for such compounds as 2-mercaptoimidazole assumes some importance.

SUMMARY

Because of the difficulty in evaluating the potency of antithyroid compounds according to the response to treatment of patients with thyrotoxicosis, a new method for assay of these drugs in normal human subjects is presented. This method depends upon modification of a predictable pattern of accumulation of radioactive iodine by the thyroid gland as detected by serial counts with an externally placed Geiger-Muller counter. The results of assay of 32 compounds in 90 subjects are given. The assay values are in most instances considerably different from those obtained by previous methods which depended upon the goitrogenic effects in animals such as the

rat and the chick. There is much closer agreement between the results by this method and those obtained from clinical trials, though some discrepancy, real or apparent, still exists. By this method some of the more active compounds tested were found to have the following potencies. Thiouracil was used as the reference standard and assigned an activity of 1.0: 2-mercaptoimidazole (10); thiocarbamylthioglycollic acid (2.5); 2-thiohydantoin (2.5); 2-mercaptothiazoline (2.5); 2-aminothiazole (2.5); 6-methylthiouracil (2); thiobarbital (2); 2-mercaptobenzimidazole (2.5); 3-mercapto-1,2,4-triazole (2); 2-mercapto-5-amino-1,3,4-thiadiazole (2); 2-mercapto-4-methylthiazole (1); thiouracil (1); thiourea (1); 6-n-propylthiouracil (0.75).

REFERENCES

ASTWOOD, E. B.: *J. Clin. Endocrinol.* 5: 345. 1945.
ASTWOOD, E. B., A. BISSELL AND A. M. HUGHES: *Endocrinology* 37: 456. 1945.
ASTWOOD, E. B., AND M. M. STANLEY: *West. J. Surg.* 1947 (In press).
BARTELS, E. C.: *J.A.M.A.* 129: 932. 1945.
BARTELS, E. C.: *Ann. Surg.* 1947 (In press); *West. J. Surg.* 1947 (In press).
BYWATER, W. B., D. A. MCGINTY AND N. D. JENESEL: *J. Pharmacol. and Exper. Therap.* 85: 14. 1945.
HAMILTON, J. G., AND M. H. SOLEY: *Am. J. Physiol.* 127: 557. 1939.
HAMILTON, J. G., AND M. H. SOLEY: *Am. J. Physiol.* 131: 135. 1940.
HERCUS, C. E.: *New Zealand M. J.* 45: 324. 1946.
HERTZ, S., A. ROBERTS AND W. T. SALTER: *J. Clin. Investigation* 21: 25. 1942.
LEYS, D.: *Lancet* 248: 461. 1945.
MCGINTY, D. A., R. W. Rawson and associates: Personal Communication. 1947.
MCGINTY, D. A., AND W. G. BYWATER: *J. Pharmacol. and Exper. Therap.* 84: 342. 1945.
MCGINTY, D. A., AND W. G. BYWATER: *J. Pharmacol. and Exper. Therap.* 85: 129. 1945.
MEULENGRACHT, E., AND K. SCHMITT: *Acta. med. Scandinav.* 122: 294. 1945.
PERRAULT, M., AND D. BOVET: *Lancet* 250: 731. 1946.
POATE, H. R. G., AND S. L. SPENCER: *M. J. Australia* 1: 493. 1946.
REVENO, W.: *J.A.M.A.* 133: 1190. 1947.
VANDERLAAN, J. E., AND W. P. VANDERLAAN: *Endocrinology.* 40: 403. 1947.
VANDERLAAN, W. P., AND A. BISSELL: *Endocrinology* 38: 308. 1946.
WILLIAMS, R. H.: *Arch. Int. Med.* 1947 (In press).
WILSON, A.: *Lancet* 250: 640. 1946.

RESPONSE OF THE ALKALINE PHOSPHATASE OF THE ADRENAL CORTEX OF THE MOUSE TO ANDROGEN[1]

HERBERT ELFTMAN

From the Department of Anatomy, College of Physicians and Surgeons, Columbia University

NEW YORK, N. Y.

ALKALINE phosphatase is a prominent constituent of the adrenal cortex of the adult male mouse but is present in only minimal quantity in the female (Elftman, 1947). This sexual dimorphism suggests the possibility that alkaline phosphatase may appear in the adrenal cortex in response to androgen. Definite evidence in favor of this relationship was obtained from the experiments which are reported here.

METHODS

Mice of the Swiss albino strain were used in all of these experiments. They comprised five groups: 1) Normal adults, 7 males and 5 females; 2) Normal immature animals, 7 males and 4 females; 3) Adult castrates, 3 males and 4 females; 4) Adult castrates treated with testosterone propionate,[2] 9 males and 8 females; 5) Immature mice treated with testosterone propionate, 12 males and 14 females.

The testosterone propionate was dissolved in sesame oil in such concentration that the daily dose was contained in 0.05 ml. for the immature animals and in 0.05 or 0.1 ml. for the adults. The mice were injected subcutaneously and autopsy followed 24 hours after the last injection.

The animals were killed with illuminating gas. The left adrenal was fixed over-night in chilled 80% ethyl alcohol with subsequent embedding in paraffin. The right adrenal was fixed in 10% neutral formol-saline. The alcohol-fixed adrenals were cut into sections 5–7 micra in thickness. For the demonstration of alkaline phosphatase the sections were incubated at 37° C. for 3 hours in a 1% sodium glycerophosphate substrate according to the technique of Gomori (1941) with the addition of 0.01M magnesium sulphate. The sections were not counterstained, in order to allow critical observation of the distribution of the cobalt sulfide. They were cleared in gasoline and mounted in clarite. Parallel sections were stained with hematoxylin and eosin.

Received for publication May 23, 1947.

[1] Aided by a grant, administered by Dr. Philip E. Smith, from the Rockefeller Foundation, New York.

[2] The testosterone propionate used in this work was supplied through the courtesy of CIBA Pharmaceutical Products, Inc., Summit, New Jersey.

OBSERVATIONS

Normal adults.

The distribution of alkaline phosphatase in the normal adult male is shown in Figures 2a and 2b. A marked concentration of the enzyme, as indicated by the deposition of cobalt sulfide, is found in both the fasciculate and the reticular zones. It is present in both the cytoplasm and the nucleus, with the reaction in the nucleus being somewhat more pronounced. The concentration, both nuclear and cytoplasmic, tends to be lower in the sub-glomerulosal portion of the fasciculata than in its inner portion. The glomerulosa offers a significant contrast to the rest of the cortex in that it gives no evidence of alkaline phosphatase under the conditions of incubation employed.

The adult female differs from the male, as may be seen in Figures 1a and 1b. The fasciculata and reticularis do not give the reaction for alkaline phosphatase. Although it is possible that more extended incubation might show the presence of the enzyme in minimal quantities, the contrast with the male would be no less striking. The glomerulosa of the female resembles that of the male in the absence of alkaline phosphatase, except for very occasional scattered regions in some individuals, in which a few cells show a positive reaction. The presence of alkaline phosphatase in the endothelium serves to accentuate its absence from the cells of the cortex.

Gonadectomized adults.

Castration of the adult male (Figures 3a and 3b) results in the disappearance of the alkaline phosphatase normally present in the adrenal cortex. As far as this particular enzyme is concerned, the cortex of the castrated male resembles that of the normal female.

The ovariectomized female (Figure 4) shows no change from the normal female (Figure 1b) in alkaline phosphatase. The lack of this enzyme in the cortex of the female mouse is as striking after ovariectomy as it is before.

Post-natal development.

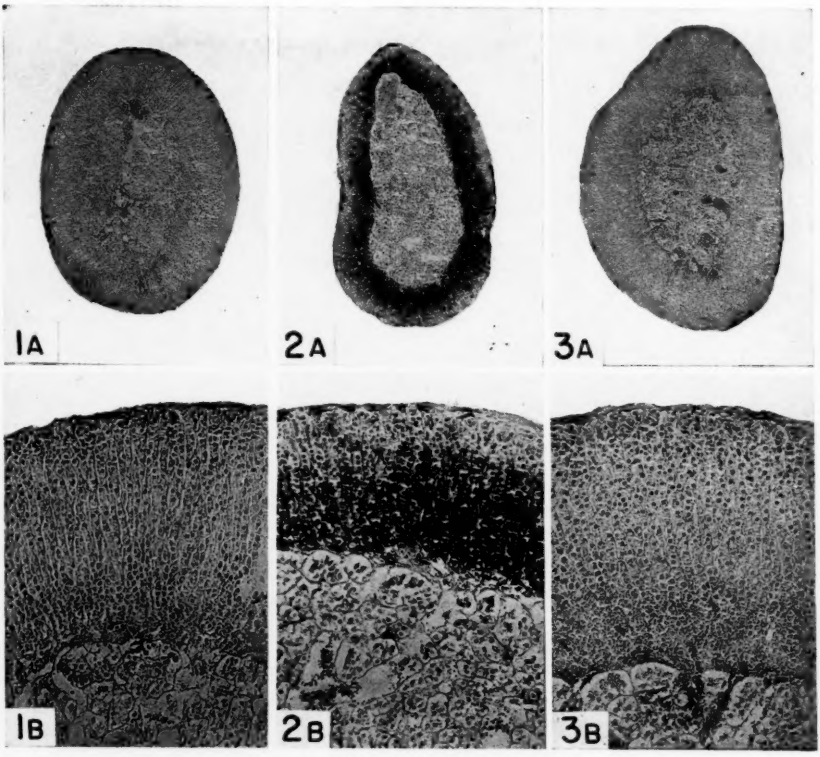
The appearance of alkaline phosphatase in the male adrenal cortex parallels the regression of the X-zone. In the normal male 24 days after birth (Figure 7) there is no evidence of the enzyme, nor is it present in the cortex at earlier post-natal stages. At 30 days, alkaline phosphatase is present in minor amounts. The adrenal cortex of the male at 42 days has achieved not only the adult content of alkaline phosphatase but also regression of the X-zone.

Female mice ranging in age from 7 to 42 days give no evidence of alkaline phosphatase in the adrenal cortex. In this they resemble the adult females.

Gonadectomized adults treated with testosterone propionate.

The administration of testosterone propionate to the castrated

Photomicrographs of the adrenal of the mouse to show the distribution of alkaline phosphatase.



FIGS. 1a (×22) and 1b (×100). Normal adult female. No evidence of alkaline phosphatase except in the endothelium and in occasional cells of the capsule.

FIGS. 2a (×22) and 2b (×100). Normal adult male. Alkaline phosphatase is abundant in the fascicular and reticular zones of the cortex.

FIGS. 3a (×22) and 3b (×100). Adult male castrated 33 days before autopsy. The alkaline phosphatase of the cortex has disappeared.

adult male results in the reappearance of alkaline phosphatase in the adrenal cortex. After 10 daily subcutaneous doses of 250 μg (Figure 6) the cortex presents the distribution of enzyme characteristic of the normal adult male. A maximum response in both cytoplasm and nucleus of the outer portion of the fasciculata is obtained after 2 daily injections of 250 μg but after this shorter time the innermost portion of the fasciculata and the reticularis do not show an increase in the enzyme. A similar distribution is obtained after 2 daily doses of 100 μg, but the reaction is somewhat less intense.

The ovariectomized female responds to testosterone propionate in the same manner as the male castrate. The distribution of alkaline

Photomicrographs (×100) of the adrenal cortex of the mouse to show the distribution of alkaline phosphatase.

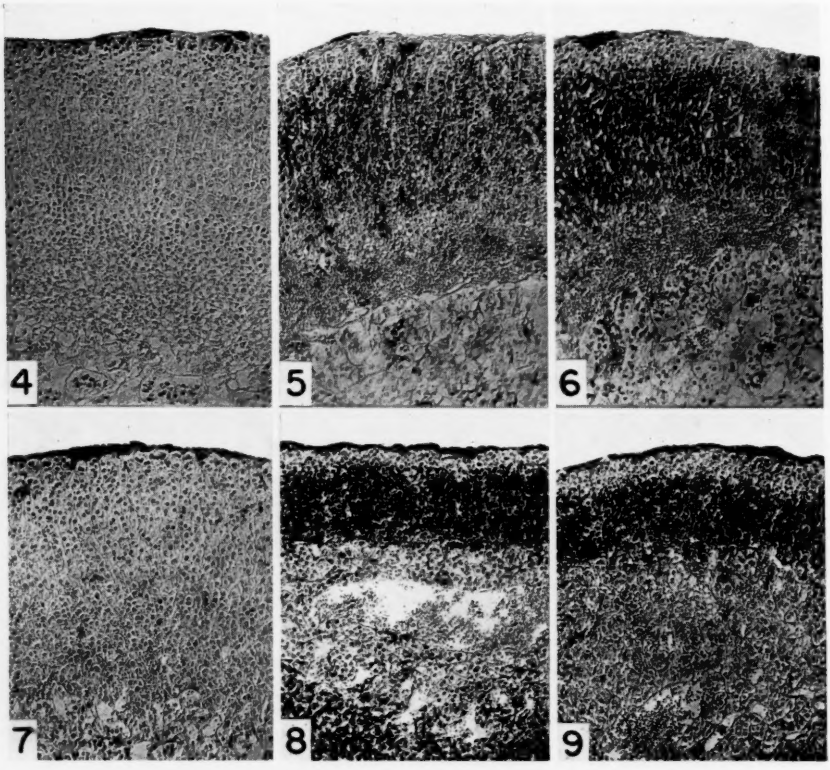


FIG. 4. Adult ovariectomized female. Absence of alkaline phosphatase as in normal female.

FIG. 5. Adult ovariectomized female after 10 injections of 250 μg of testosterone propionate. Alkaline phosphatase has appeared in a distribution similar to that of the normal male but with a lower concentration, especially in the cytoplasm.

FIG. 6. Adult castrated male after 10 injections of 250 μg of testosterone propionate. The alkaline phosphatase which disappears after castration (Fig. 3b) has been restored.

FIG. 7. Normal immature male, 24 days old. Alkaline phosphatase has not yet appeared in the cortex.

FIGS. 8 and 9. Immature female and male respectively, 22 days old, after 5 daily injections of 250 μg of testosterone propionate. The distribution and concentration of alkaline phosphatase are similar to those of the normal adult male.

phosphatase after 10 daily doses of 250 μg (Figure 5) is essentially similar to that of the normal adult male but the concentration is somewhat lower, especially in the cytoplasm. It consequently provides a marked contrast to the normal adult female (Figure 1b) and the ovariectomized untreated female (Figure 4). The response to

lower dosages and shorter periods of administration in the ovariectomized female is similar to that in the castrated male.

Immature animals treated with testosterone propionate.

Although alkaline phosphatase is not present in the adrenal cortex of immature female mice or in males for at least the first 24 days (Figure 7), it is possible to cause its appearance by administering testosterone propionate. Both the female (Figure 8) and the male (Figure 9) when autopsied at 22 days of age following 5 daily injections of 250 μg show an alkaline phosphatase distribution similar to that of the normal adult male. Two daily doses of 100 μg suffice to produce a marked response, strongest in the outer portion of the fasciculata.

DISCUSSION

The presence of a high concentration of alkaline phosphatase in the fascicular and reticular zones of the adrenal cortex of the adult male mouse contrasts sharply with the absence or minimal quantity of the enzyme in the adult female. This difference between the sexes suggests that alkaline phosphatase in the adrenal cortex is either mobilized by androgen or suppressed by estrogen. The latter possibility is ruled out by the fact that ovariectomy is not followed by the appearance of the enzyme. Castration of the male, however, results in the disappearance of the cortical alkaline phosphatase. This would indicate that the continued presence of the enzyme in this location is dependent on the presence of androgen.

Further correlation between androgen and cortical alkaline phosphatase is provided by the parallelism between the time of first appearance of the enzyme in the male and the regression of the X-zone.

That the disappearance of the X-zone is due to the action of androgen has been conclusively proved by Howard (1927), Deanesly (1928), Martin (1930), Deanesly and Parkes (1937) and subsequent investigators. The absence of alkaline phosphatase in the adrenal cortex of the immature mouse until its appearance in the male during the regression of the X-zone is consequently further evidence in favor of its mobilization by androgen.

Direct evidence of the power of an androgen to mobilize alkaline phosphatase in the adrenal cortex is provided by the administration of testosterone propionate. This treatment evokes cortical alkaline phosphatase not only in the castrated adult male but also in the ovariectomized female and in immature animals of both sexes.

The sexual dimorphism in the distribution of alkaline phosphatase in the adrenal cortex of the adult Swiss albino mouse adds a qualitative feature to the previously known quantitative differences between the adrenals of the two sexes. In other animals cortical alkaline phosphatase may not be so completely under androgen control. Preliminary investigations of the adrenal cortex of the rat and

of the rabbit indicate that in them also the male has a higher concentration of cortical alkaline phosphatase than does the female but with the difference less highly accentuated than in the mouse.

The absence of alkaline phosphatase from the glomerulosa of the mouse adrenal offers further histochemical support for the distinction between glomerulosa and fasciculata, made originally on morphological grounds. The fact that the outer zone of the fasciculata gives a phosphatase response to shorter periods of testosterone propionate treatment than does the inner zone may indicate either differential reactivity to the hormone or actual cell migration.

The fact that alkaline phosphatase is mobilized in the adrenal cortex by androgen recalls the similar function exercised by estrogen in the epithelium and circular muscle of the uterus (Atkinson and Elftman, 1946, 1947). Other examples of increases in alkaline phosphatase after steroid hormone administration have been recorded with less accurate localization by other than histochemical methods. Among these are the effect of testosterone on rat femurs (Williams and Watson, 1941) and the renal response to desoxycorticosterone (Kutscher and Wüst, 1942; Folley and Greenbaum, 1946). Ability to mobilize alkaline phosphatase is one of the properties of these hormones and may be a significant link in some of their physiological actions.

Although the experiments reported here prove that androgen mobilizes alkaline phosphatase in the adrenal cortex, they do not preclude the participation of the hypophysis as an intermediary mechanism. The effects of hypophyseal hormones on the cortical content of constituents other than alkaline phosphatase have been extensively studied, most recently by Sayers, Sayers, Fry, White and Long (1944); Carreyett, Golla and Reiss (1945); and Deane and Greep (1946). Consideration must consequently be given to the possibility that the mobilization of cortical alkaline phosphatase is mediated by the hypophysis. Preliminary experiments indicate, however, that in hypophysectomized animals androgen is still able to increase the alkaline phosphatase of the adrenal cortex.

SUMMARY

Alkaline phosphatase is present in marked concentration in the nuclei and cytoplasm of the fascicular and reticular zones of the adrenal cortex of the adult male mouse. It is absent, or present only in traces, in the normal female, the ovariectomized female, the castrated male and the immature male. Its first appearance in the male is concurrent with the regression of the X-zone.

Treatment with testosterone propionate results in the appearance of alkaline phosphatase in the adrenal cortex of the castrated male, the ovariectomized female and immature animals of both sexes.

The alkaline phosphatase of the adrenal cortex of the mouse is under the control of androgen. The glomerulosa differs from the fasciculata in its inability to acquire this enzyme through androgenic stimulation. The mobilization of cortical alkaline phosphatase by androgen provides another link in the chain of reactions associated with the physiology of androgens and of the adrenal cortex.

REFERENCES

ATKINSON, W. B., AND H. ELFTMAN: *Proc. Soc. Exper. Biol. & Med.* 62: 148. 1946.
ATKINSON, W. B., AND H. ELFTMAN: *Endocrinology* 40: 30. 1947.
CARREYETT, R. A., Y. M. L. GOLLA AND M. REISS: *J. Physiol.* 104: 210. 1945.
DEANE, H. W., AND R. O. GREEP: *Am. J. Anat.* 79: 117. 1946.
DEANESLY, R.: *Proc. Roy. Soc. B*103: 523. 1928.
DEANESLY, R., AND A. S. PARKES: *Quart. J. Exper. Physiol.* 26: 393. 1937.
ELFTMAN, H.: *Anat. Rec.* 97: 331. 1947.
FOLLEY, S. J., AND A. L. GREENBAUM: *Biochem. J.* 40: 46. 1946.
GOMORI, G.: *J. Cell. & Comp. Physiol.* 17: 71. 1941.
HOWARD, E.: *Am. J. Anat.* 40: 251. 1927.
KUTSCHER, W., AND H. WIST: *Hoppe-Seyl. Z.* 273: 235. 1942.
MARTIN, S. J.: *Proc. Soc. Exper. Biol. & Med.* 28: 41. 1930.
SAYERS, G., M. A. SAYERS, E. G. FRY, A. WHITE AND C. N. H. LONG: *Yale J. Biol. and Med.* 16: 361. 1944.
WILLIAMS, H. L., AND E. M. WATSON: *Endocrinology* 29: 258, 1941.

GONADOTROPHIC EFFECTS OF EXOGENOUS SEX HORMONES ON THE TESTES OF SPARROWS[1]

CARROLL A. PFEIFFER
Department of Anatomy, Yale University

THE MECHANISM by which exogenous androgens act to maintain spermatogenesis or to cause spermatogenesis to go to completion in the regressed testis is not understood. It would seem that the androgen does not act through the hypophysis in mammals since it maintains spermatogenesis in the hypophysectomized rat (Nelson, 1941; Walch, Cuyler and McCullach, 1934) and stimulates spermatogenesis in ground squirrels hypophysectomized during the non-breeding season (Wells, 1942). Until the present investigation was completed,[2] the only evidence that androgens stimulate the bird testis had been presented by Chu (1940) who found testis stimulation in 3 hypophysectomized pigeons. On the other hand, it had been reported for fowl (Morató-Manaro, Albrieux and Bruño, 1938) and Starlings (Burger, 1944) that androgen caused regression of the testes. However, recently androgen was shown to cause an increase in the size of the dove testis (Lahr and Riddle, 1944) and to stimulate spermatogenesis in Starling testes which had reached the spermatocyte stage before treatment was begun (Burger, 1945).

It has been mentioned on several occasions that androgens injected into female birds cause stimulation of the oviducts (Noble and Wurm, 1940; Pfeiffer, Kirschbaum and Gardner, 1940; Pfeiffer and Kirschbaum 1941). In the sparrow it was usually assumed that the androgen produced this effect indirectly, causing the hypophysis to release sufficient quantities of gonadotrophic hormones to produce a slight stimulation of the ovaries, which then secreted enough estrogen to affect the oviducts (Pfeiffer and Kirschbaum, 1941). However, Witschi (1943) has reported that testosterone propionate will stimulate the oviducts of the castrated sparrow, although 60 μg. is required to give a minimal response. This, together with the fact that estrogens have been assumed to inhibit testicular development by suppressing

Received for publication April 25, 1947.

[1] This work was aided by grants from the Fluid Research Fund of Yale University School of Medicine, The Anna Fuller Fund, The National Research Council Committee for Research in Problems of Sex (grant administered by Professor W. U. Gardner) and the International Cancer Research Foundation.

[2] This material was presented before the Association for the Study of Internal Secretions at the meeting held in Chicago, June 1943. See abstract: Pfeiffer, C. A.: *Endocrinology* 35: 202, 1943.

the hypophysis (Pfeiffer and Gardner, 1938), raises some doubt as to whether androgen can cause the hypophysis to secrete gonadotrophic hormones in the bird. Therefore, it was decided to investigate rather thoroughly the whole question of the effects of exogenous sex hormones on the testis of the sparrow. Since it would be difficult to hypophysectomize such a small bird, advantage was taken of the fact that the hypophysis is easily stimulated at the beginning of the normal breeding season and is quite unresponsive during the non-breeding season (Kirschbaum, Pfeiffer, van Heuverswyn and Gardner, 1939).

MATERIAL AND METHODS

A total of 51 experimental and 30 control English sparrows (Passer domesticus) were used for this investigation. They were trapped in nature and maintained in wire cages 3×3×4 feet in dimension. They were fed a dry mixture of equal parts of finely cracked grain (chick) and poultry growing mash. Fresh food and water were supplied continuously. The estradiol benzoate and testosterone propionate[3] were dissolved in sesame oil in such concentration that the desired amount of hormone could be injected in 0.05 cc. of oil. The pregnant mare serum (PMS)[4] was injected as prepared by the commercial company. All animals were injected daily, the hormones being given intramuscularly in the breast region, using alternate sides on alternate days.

For convenience the specific treatment is given in the section describing the results of that treatment. The ages of the birds were not known definitely but could be estimated fairly accurately since most of the birds were trapped shortly after leaving the nest. The time that elapses after the bird leaves the nest can be readily estimated by the size of the lateral pouches of the bill. Birds with extremely large pouches are termed 'fledglings,' and could not have been out of the nest more than a week. Whenever fledglings were used in the experiments, they were allowed to become acclimated to the cages for a few days before injections were started. The term 'young juvenal' is applied after the lateral pouches have regressed somewhat but before the throat feathers of the male darken. The term 'juvenal' is used from the time the throat feathers of the male begin to darken at their bases until the juvenal plumage has completely given way to the adult sex plumage. The term 'young adults' applies from the time the birds assume complete adult plumage until the beginning of the first breeding season.

Laparotomies were performed at the start of each experiment and the length and width of the testes were determined. The testes were measured by adjusting calipers to them and then transferring the calipers to a millimeter rule. Laparotomies and measurements of the testes were repeated every two weeks during the experiment. A somewhat more accurate measurement was made at autopsy when the testes were removed.

---

[3] The estradiol benzoate and testosterone propionate were supplied by the Schering Corporation through the courtesy of Dr. E. Schwenk.

[4] The pregnant mare serum used was Gonadin, the highly purified gonadotrophic hormone from pregnant mare serum. It was supplied by the Cutter Laboratories through the courtesy of Dr. D. H. Wonder.

EXPERIMENTAL

Testicular response in sparrows receiving estrogens. Six adult males received 5.0 μg. of estradiol benzoate daily for two weeks during the period of testis enlargement which precedes the breeding season. The average size of the testes at the start of the experiment was 6.0 mm. (fig. 1) in greatest diameter (range 5.0 to 7.0 mm.). After 2 weeks of treatment all of the testes had decreased to an average size of 1.0 mm. (fig. 2) in greatest diameter (range 0.5 to 1.7 mm.). The individual

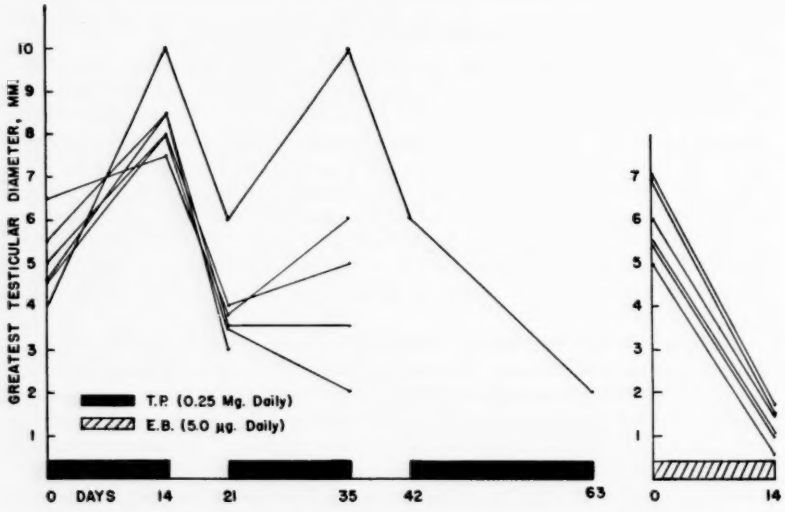


CHART 1. The graph at the right shows the marked regression of the testes which occurs when sparrows are treated for two weeks with 5.0 μg. of estradiol benzoate daily. The graph at the left illustrates the fluctuations in size of the testes of sparrows treated intermittently with 0.25 mg. of testosterone propionate daily. The solid blocks along the abscissa designate the periods when androgen was administered; the spaces left blank indicate the periods when treatment was suspended.

figures are given in chart 1. The decrease was made more striking by the fact that during this period the testes of untreated birds were still undergoing their normal seasonal enlargement. The same daily dosage of estradiol benzoate administered to 8 juvenal and 8 fledgling male birds produced no difference in testis size from that of control birds of the same age, nor did it cause any histologically detectable evidence of either stimulation or regression of the testis (compare figs. 3 and 4). Since this failure of the estrogen to affect the testes of fledgling birds indicates that estrogen does not act directly on the testis, it would seem evident that its effect when the testes are undergoing normal seasonal enlargement (compare figs. 1 and 2) is accomplished through inhibition of the hypophysis.

Testis response in adult sparrows receiving androgens. Seven adult males were given 0.25 mg. of testosterone propionate daily for periods of from 4 to 11 weeks. The injections were begun early in the period of normal seasonal enlargement of the testes, when the bills had just begun to show pigmentation. The effect of this treatment on the size of the testes is shown in chart 2. It may be seen that when the testes were less than 2.0 to 3.0 mm. in greatest diameter at the begin-

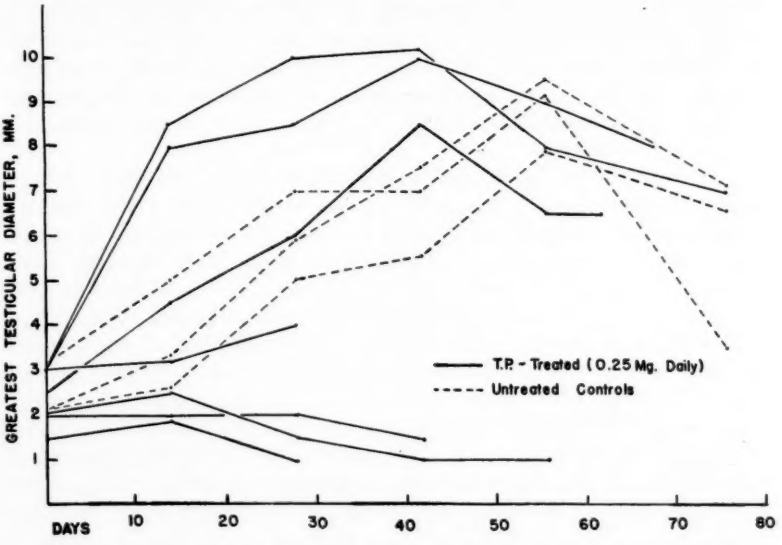


CHART 2. In this chart the rate of enlargement of the testes of sparrows treated with 0.25 mg. of testosterone propionate daily is shown in comparison with the normal seasonal rate of enlargement. Note that when the testes are 3.0 mm. in greatest diameter at the start of androgen treatment they enlarge rapidly; when they are around 2.0 mm. or less they regress to the size characteristic of the testes of sexually quiescent birds.

ning of treatment the androgen had no gonadotrophic effect upon them but instead evidently suppressed the hypophysis. However, when the testes were larger than 3.0 mm. in greatest diameter the androgen caused them to enlarge quite rapidly (charts 1 and 2). There was a certain amount of overlap in the size at which the testes would respond to stimulation by the androgen which may have been due partly to the difficulty of making accurate measurements of the small intact testes. When the testes were sufficiently developed to be stimulated by androgen, they grew at an appreciably faster rate than did the controls which received no treatment. They reached almost their maximum size in the first two weeks, continued enlarging at a slower rate during the next 4 weeks, and then gradually decreased in size. This falling off at the end of the experiment presumably is not a

direct effect of the androgen since it is also evident in the controls. It is probably due to the rapid liberation of mature sperm.

Histological study showed the development of the testes in the testosterone propionate treated birds to be similar to that in the untreated birds. The only difference noted was that in the treated birds complete spermatogenesis was attained earlier and there was an appreciable intertubular edema. At 2 weeks spermatogenesis was at about the same stage as that seen in the controls at 4 weeks (fig. 5). There were a few enlarged fibroblasts with fairly distinct cytoplasm in the interstitial spaces but no true Leydig cells. The testes that had not reached the stage at which they could be stimulated with androgen showed the typical gradual regression which results from inhibition of the hypophysis (fig. 6). Lymphocytes were not present in appreciable numbers.

Intermittent treatment with androgen. Since the androgen apparently suppressed the hypophysis in the birds whose testes had not developed far enough to respond to androgen treatment it would seem probable that it also inhibited the hypophysis in the birds whose testes did respond and therefore that the response in the latter birds was due to a direct action of the administered androgen on the testis. To further test this idea 6 males were given 0.25 mg. of testosterone propionate daily for 2-week periods with a week of rest between each period (see chart 1). Birds were chosen for this experiment whose testes were well within the size range at which they could be expected to respond readily to androgen treatment. At the start of the experiment the testes averaged 5.0 mm. in greatest diameter (range 4.0 to 6.5 mm.). After 2 weeks of treatment the average size was 8.5 mm. (range 7.5 to 10.0 mm.). Following a week of rest from the treatment the average size of the testes had fallen to 3.95 mm. (range 3.0 to 6.0 mm.). At the close of the second 2-week period of treatment the

---

FIG. 1. Condition of the testis during the period of testicular enlargement which precedes the breeding season. This testis measured approximately 6.0 mm. in greatest diameter. Note spermatocytes and a few spermatids in the seminiferous tubules, and fibroblasts in the intertubular spaces.

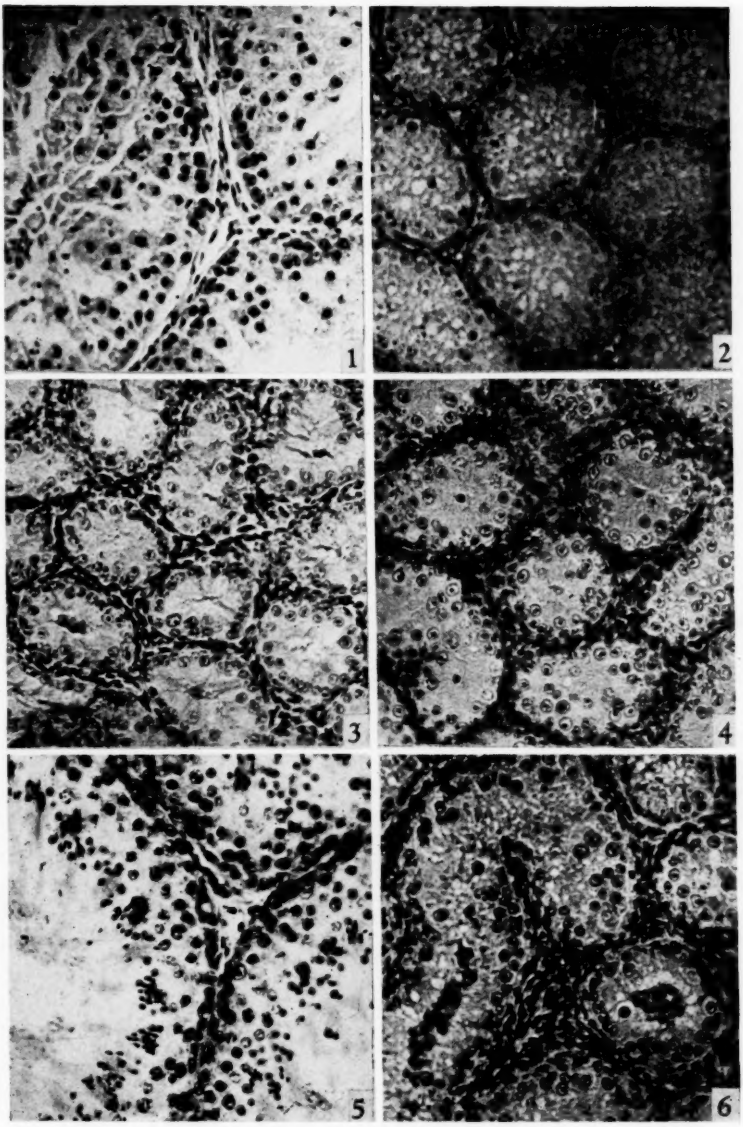
FIG. 2. Regression of the testis following treatment with 5.0 μg. of estradiol benzoate daily for two weeks. At the start of treatment this testis was at the stage of development illustrated in figure 1. Note that only a few spermatogonia and Sertoli cells remain.

FIG. 3. Testis of a fledgling sparrow which was used as a control.

FIG. 4. Testis of a fledgling sparrow which was treated for two weeks with 5 μg. of estradiol benzoate daily. Note that there is neither stimulation nor regression of the testis.

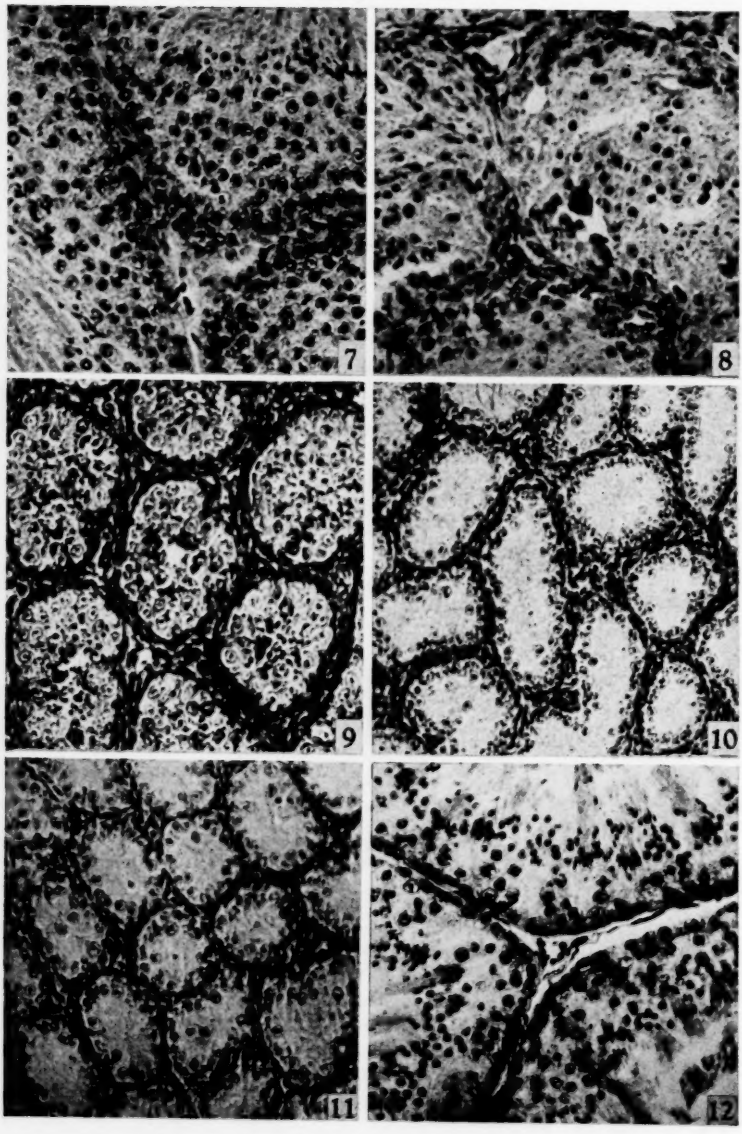
FIG. 5. Complete spermatogenesis in the testis of a sparrow which had received 0.25 mg. of testosterone propionate daily for two weeks. This condition can be maintained by injected androgen for at least 80 days. At the start of treatment this testis measured 3.0 mm. in greatest diameter.

FIG. 6. Regression of the testis of a sparrow which had received the same treatment as the one whose testis is shown in figure 5, except that treatment was begun when the testis measured 2.0 mm. in greatest diameter.



FIGS. 1–6

All figures are photomicrographs of sparrow testes stained with Ehrlich's hematoxylin and triosin. ×280.



FIGS. 7–12

average testis size was 5.3 mm. (range 2.0 to 10.0 mm.). In only one bird did the testes return to the size attained after the first stimulation with androgen (10.0 mm.). This bird was allowed to rest for another week during which time the testes again regressed to the size (6.0 mm.) they were after the first rest period. Testosterone propionate was once more administered, the treatment this time being continued for 3 weeks, but the testes continued to regress so that at the close of this period they measured only 2.0 mm. in greatest diameter.

The individual data on these birds, presented in chart 1, indicate that after a period of stimulation and a week of regression the testes must be between 3.0 and 4.0 mm. in greatest diameter to respond to a second treatment with androgen, as contrasted to the size (2.0 to 3.0 mm.) at which the testes may respond to a first treatment. Moreover, there was a great difference in the degree of response. In the case of the single bird which received a third series of injections, the testes failed to respond even though they were 6.0 mm. in length at the time the third treatment was begun. These differences in response to intermittent treatment with androgen can be understood on the basis of the histology of the testes.

At the end of the first 2 weeks of treatment with testosterone propionate the testes could not be distinguished histologically from those of normally enlarging testes at the beginning of sexual activity. Spermatogenesis was just beginning to go to completion (fig. 7). After a week of interruption in the hormone treatment the testes showed the histological changes typical of regression (fig. 8). Sperm and spermatids had been sloughed almost completely, and the secondary spermatocytes had begun to undergo degenerative changes; they were losing their cohesiveness, the cytoplasmic granules had

FIG. 7. Control for figure 8. This testis was removed after the sparrow had received 0.25 mg. of testosterone propionate daily for two weeks. Note spermatogenesis going to completion.

FIG. 8. Seminiferous tubules of the second testis of the sparrow which furnished that shown in figure 7. This testis was left in place 7 days after the first was removed, during which time no hormone treatment was given. Note the marked regression which follows the cessation of androgen treatment.

FIG. 9. Testis of the single bird which received intermittent androgen treatment for 63 days. The changes in testis size which accompanied each period of treatment are shown in chart 1. Note the extreme regression of the germinal epithelium and the accumulation of cellular debris.

FIG. 10. Testis of an untreated young juvenal sparrow.

FIG. 11. Testis of a young juvenal sparrow after 3 weeks of treatment with 0.25 mg. of testosterone propionate daily. Note that there is neither stimulation nor regression.

FIG. 12. Testis of a young juvenal sparrow which was treated with 25.0 I.U. of PMS daily until the testis began to release androgen (10 days) and then was given 0.25 mg. of testosterone propionate daily for the next 40 days. Note that complete spermatogenesis was induced and maintained. This testis is identical with that of the normal bird during the breeding season.

become less clearly defined and the nuclei had become pycnotic. The testes were hyperemic, the cells in the interstitial spaces were mainly fibroblasts, but an appreciable number of lymphocytes was present. There were a few cells resembling histiocytes which were thought to be stages in the formation of lymphocytes.

The testes showed considerable variation in the extent of these degenerative changes at the end of the period of rest. These differences were reflected in the response of the testes to the second 2 weeks of treatment. The most degenerate continued to regress, as was also evidenced by a decrease in size (chart 1). Others increased in size, due principally to an increase in intratubular fluid. However, in most of the birds there was a definite stimulation of spermatocyte formation, while, at the same time, there usually was evidence that the more mature stages of spermatogenesis were being sloughed. In only the single bird whose testes reached a size of 10.0 mm. was there complete spermatogenesis at the end of the second period of treatment, and even here all stages beyond the primary spermatocytes were beginning to show evidence of slight pycnosis. After another week of rest a rapid regression was evidenced by a decrease in testis size. This regression continued during subsequent androgen treatment so that after 3 weeks of continuous treatment the tubules had returned to the size characteristic of the non-breeding male. However, the tubules still contained some debris (fig. 9). There was also evidence of fatty degeneration of cells, and an appreciable amount of yellow or brown pigment was present in both the cells in the lumina of the tubules and the cells of the interstitium. The cells in the interstitial spaces behaved in all cases as they do in normal growth and regression of the testes. There was no evidence of true Leydig cell development.

Testis response of juvenal males receiving testosterone propionate. Eight young juvenal males received 0.25 mg. of testosterone propionate daily for 3 weeks without showing any evidence of testicular stimulation (compare figs. 10 and 11), indicating that androgen can neither stimulate the hypophysis to release gonadotrophins nor stimulate the immature testis directly at this age. Therefore, 8 fledglings were started on 25 I.U. of PMS daily until the bills began to darken (10–14 days) showing that the testes were releasing androgen. In previous experiments it was shown that this occurred when the testes were around 2.0 to 3.0 mm. in length. At this point 0.25 mg. of testosterone propionate daily were substituted for the PMS, and the testes continued to develop for at least 40 days, at which time the last animal was autopsied. This development was identical in all respects with that found in adult animals whose testes were over 3.0 mm. in length at the time androgen treatment was started (compare figs. 5 and 12).

DISCUSSION

It seems clear from the above findings that androgen stimulates complete spermatogenesis in the sparrow if the testes are already 3.0

mm. or more in greatest diameter. Androgen does not, however, stimulate the hypophysis to release appreciable amounts of gonadotrophic hormones. In fact it seems more likely that, in the dose given here at least, it inhibits the release of these substances. This is suggested by the fact that the testes are inhibited by androgen if they have not been stimulated by the hypophysis to a certain stage of development (between 2.0 and 3.0 mm. in greatest diameter) and by the finding that when the testes are greatly stimulated by androgen, the cessation of injections is followed by very rapid regression even though the hypophyses of control sparrows are continuing to release gonadotrophic substances at a high rate. That androgen inhibits the hypophysis has also been indicated by experiments on fowl (Moratό-Manaro, Albrieux and Bruño, 1938) and Starlings (Burger,1944). These experiments failed, however, to demonstrate a gonadotrophic effect of androgen.

The apparent discrepancy between the Starling (Burger, 1944) and sparrow is probably not a real one. The two experiments were not designed to show the same thing. If we assume that the testes of the Starlings on February 17 had not enlarged to a stage comparable to the 2.0 to 3.0 mm. size in the sparrow, then it would be expected that only a suppression of the hypophysis and therefore a regression of the testes would occur, just as Burger observed. The slight enlargement occurring after 26 to 36 days would be, as this author interpreted, a slight over-riding of the androgen suppression of the hypophysis by the 14 hours of lengthened daylight. In the second experiment the testes were evidently of sufficient size to be stimulated by the androgen, but the stimulus of 14 hours additional light probably caused maximal development of the testes so that any effect of the androgen was masked. More recent experiments (Burger, 1945) indicate that the Starling does respond quite similarly to the sparrow.

The findings in fowl are not so easily explained. It is reported that the testes of 3 months old Leghorn cocks are inhibited when androgen is injected (Moratό-Manero, Albrieux and Bruño, 1938), although at this age they should be at about the stage at which the testes can be stimulated in the sparrow. If a difference between these two species of birds does exist it may be due to the fact that the fowl has a continuous breeding season, whereas the sparrow is a seasonal breeder. The evidence from mammals is that there is a difference between seasonal and non-seasonal breeders in the response of the testis to androgen (Moore and Morgan, 1942; Wells, 1943b). It has been thought that this difference may be due to a maturity factor since the same conditions hold for gonadotrophins (Wells, 1943a). In the ground squirrel the immature testes readily form mature sperm after treatment with gonadotrophins (Wells, 1943b) and androgens (Wells 1943a), but with continuous breeders, such as the rat, injected gonadotrophins and androgens (Cutuley, 1941; Nelson, 1941) induce the production of mature sperm only after the testes have

reached a certain state of maturity and have regressed due to hypophyseal impairment. Conditions in the sparrow cannot, however, be adequately explained on the basis of a maturity factor alone since the testes of nestling birds respond to PMS (Kirschbaum, Pfeiffer, van Hewerswyn and Gardner, 1939), whereas androgen does not stimulate the testes until they have been brought, either by normal seasonal stimulation or by the administration of gonadotrophic hormones, to the stage at which androgen would be produced in measurable amounts.

We are thus still faced with the problem of how androgen acts to bring about complete spermatogenesis. The data in the literature, despite the many inconsistencies, suggests that spermatogenesis may not be a direct result of hormone action but rather is able to go to completion only when in a favorable environment. It would seem, therefore, that androgen may function simply to produce or maintain the proper environment, even in cases where action of the androgen is so localized as to affect only the tissues of the testis (Smith, 1944; Dvosken, 1944; Simpson, Li and Evans, 1946). In the present experiment and in that of Dvoskin (1944), scrotal physiology is ruled out, but temperature and vascularity are not completely eliminated as factors in the maintenance of a suitable environment. The finding that androgen actually hastens the regression of the cryptorchid testis of the rat (Hamilton and Leonard, 1938) is difficult to explain on any other basis. However, it does not make the role of the androgen in maintaining a suitable environment any clearer.

It is extremely interesting that the testes become capable of responding to androgen at about the time that they reach the stage at which they begin to liberate androgen in measurable amounts. This correlation is so reliable that the beginning of androgen production as evidenced by the onset of darkening of the bill has been used as a criterion to determine the time at which laporatomies should be performed and the birds started on treatment after either normal seasonal or PMS stimulation. As has been shown by Pfeiffer and Kirschbaum (1943) the testis size varies considerably at the time androgen secretion begins. In adult males free in nature and adult males that received large doses of PMS, androgen production begins when the testes are approximately 2.0 $\times$ 2.0 mm. in size, but the histological charactertistics of the testes are different under the two conditions. in the former the only activity seen in the testis is the formation of spermatocytes, while in the latter only interstitial development may occur. When the testes are stimulated with added light, they usually reach a size of 3.0 to 4.0 mm. in diameter before any measurable amount of hormone is released, but the development of the testis is histologically identical with that occurring in nature. Unfortunately all of the above conditions were not duplicated, and therefore it is impossible to say whether androgen production by the

testis and the ability of the testis to be stimulated by androgen always exactly coincide, but it is evident that they are very closely related.

The close correlation in the sparrow between the time that androgen is produced in appreciable amounts in the testis and the time at which spermatogenesis can be stimulated by exogenous androgens would suggest that the hypophysis stimulates the production of androgen by the testis and that this androgen is responsible for the completion of spermatogenesis, were it not that the histological and cytological characteristics of the testis are not in agreement with the logical endocrine concept of gonad-hypophyseal interrelationship. This concept has as its first premise that the introduction of an exogenous sex hormone into the system causes the suppression of the cells or tissues which produce that sex hormone in the gonad. In the normal breeding sparrow the only structures in the testis that can be correlated with the production of androgen are the intratubular elements (the spermatogenically active germinal epithelium), and these have been shown to be stimulated by exogenous androgen. Even if we assume that Leydig cells develop and produce the androgen, as it is possible to make them do under certain types of experimental procedures (Pfeiffer and Kirschbaum, 1943), and that these cells are altered by the rapid growth of the testis so that they are not readily identified as Leydig cells, the discrepancy still exists, since the interstitial tissue is identical whether androgen has been injected or the testis allowed to develop normally at the breeding season. Burger (1944) has also reported that the interstitial tissue of the enlarging Starling testis is not altered by injected androgen. It may be possible with special stains to identify in the normal sparrow testis, secretory cells which are not present in androgen-treated birds, but so far there is no evidence of this.

From the histological evidence and the fact that the testes of control birds regress after they have reached their maximum size, it seems fairly conclusive that the decrease in size of the testes after 40 days of androgen-treatment is due to the release of mature sperm. This agrees with the findings on both normal (Wells, 1935) and androgen-treated ground squirrels (Wells, 1943a). It is unfortunate that the present experiments were not designed to determine how long the testes could be maintained by the injection of androgen, but the oil solvent of the hormone accumulated under the skin in amounts which impaired the health of the birds before the 80th day.

SUMMARY

Injection of 0.25 mg. of testosterone propionate daily will stimulate complete spermatogenesis in the sparrow, provided the testis has attained the stage of development (2.0 to 3.0 mm. in greatest diameter) at which androgen begins to be produced in readily measurable amounts, and will maintain complete spermatogenesis for at

least 80 days. Estrogen has no stimulating effect on the testis at any stage of development. Both estrogen and androgen suppress the hypophysis of the sparrow. At any time that the hypophysis is not releasing gonadotrophic hormones in amounts sufficient to have a stimulating effect on the testis, the testis can be brought to the stage at which androgen is produced by the injection of pregnant mare serum, after which the substitution of androgen for the PMS will bring about complete spermatogenesis. It is therefore concluded that gonadotrophic hormones are essential for bringing the sparrow's testes up to the stage at which androgen is produced in measurable amounts, but that after this stage is reached androgen alone is sufficient to carry spermatogenesis to completion.

Injected androgen does not produce any visible suppression of the interstitial tissue. Therefore, it is difficult to determine both the source of the androgen in the normal testis and the way in which exogenous androgen acts to bring about complete spermatogenesis.

REFERENCES

BURGER, J. W.: *Endocrinology* 35: 182–186. 1944.
BURGER, J. W.: *Endocrinology* 37: 77–82. 1945.
CHU, J. P.: *J. Endocrinology* 2: 21–37. 1940.
CUTULEY, E.: *Anat. Rec.* 79 (Suppl. 2): 16. 1941.
DVOSKIN, S.: *Am. J. Anat.* 75: 289–327. 1944.
HAMILTON, J. B., AND S. L. LEONARD: *Anat. Rec.* 71: 105–117. 1938.
KIRSCHBAUM, A., C. A. PFEIFFER, J. D. VAN HEUVERSWYN AND W. U. GARDNER: *Anat. Rec.* 75: 249–263. 1939.
LAHR, E. L., AND O. RIDDLE: *Endocrinology* 35: 261–266. 1944.
MOORE, C. R., AND C. F. MORGAN: *Endocrinology* 30: 990–999. 1942.
MORATÓ-MANARO, J., A. S. ALBRIEUX AND W. BRUÑO: *Klin. Wchnschr.* 17: 784–785. 1938.
NELSON, W. O.: *Anat. Rec.* 79 (Suppl. 2): 48. 1941.
NOBLE, G. K., AND M. WURM: *Endocrinology* 26: 837–850. 1940.
PFIFFER, C. A., AND W. U. GARDNER: *Anat. Rec.* 72 (Suppl.): 59. 1938.
PFEIFFER, C. A., AND A. KIRSCHBAUM: *Yale J. Biol. & Med.* 13: 315–322. 1941.
PFEIFFER, C. A., AND A. KIRSCHBAUM: *Anat. Rec.* 85: 211–227. 1943.
PFEIFFER, C. A., A. KIRSCHBAUM AND W. U. GARDNER: *Yale J. Biol. & Med.* 13: 279–284. 1940.
SIMPSON, M. E., C. H. LI AND H. M. EVANS: *Endocrinology* 39: 281–285. 1946.
SMITH, P. E.: *Yale J. Biol. & Med.* 17: 281–287. 1944.
WALCH, E. L., W. K. CUYLER AND D. R. MCCULLACH: *Am. J. Physiol.* 107: 508–512. 1934.
WELLS, L. J.: *Anat. Rec.* 62: 409–447. 1935.
WELLS, L. J.: *Anat. Rec.* 82: 565–585. 1942.
WELLS, L. J.: *Endocrinology* 32: 455–462. 1943a.
WELLS, L. J.: *Surgery* 14: 436–472. 1943b.
WITSCHI, E.: *Anat. Rec.* 87: 478–479. 1943.

NOTES AND COMMENTS

THE DEVELOPMENT OF A REFRACTORY STATE TO THE ADRENOCORTICOTROPHIC HORMONE[1]

In a previous communication (Anderson, Page, Li and Ogden, 1944) it was shown that hypophysectomy caused a fall in the blood pressure of rats with renal hypertension and that the giving of adrenocorticotrophic hormone (ACT) restored the hypertension to the prehypophysectomy level. Subsequent observations have shown, however, that the renal hypertension could not be maintained in the hypophysectomized rat after 10 days despite continued injections of ACT. It is the purpose of this communication to report the data on the development of a refractory state to this hormone.

METHODS

Adult rats of both sexes were used. The arterial hypertension was produced by partial occlusion of the left renal artery, in the manner described by Wilson and Byrom (1939). Systolic blood pressure was measured by the tail plethysmograph method of Williams, Harrison and Grollman (1939). Hypophysectomy was performed by the technique first described by P. E. Smith (1927). The ACT was prepared by the method of Li, Evans and Simpson (1943). This preparation has been shown by electrophoretic, sedimentation and solubility studies to be a single substance.

EXPERIMENTAL DATA

Partial occlusion of the left renal artery was done on 6 rats, which were approximately 2 months of age. Blood pressure readings were made 2 to 3 times a week. After 6 to 10 weeks, when the blood pressure had reached hypertensive plateau, the animals were hypophysectomized. Following this operation the animals were placed in a temperature-controlled room, maintained at 28° C., where they remained throughout the experimental period. The average blood pressure reading before hypophysectomy was 160 mm. Hg. Immediately following removal of the pituitary the blood pressure began to fall, and within 2 weeks had levelled off to an average of 123 mm. Hg. One month after hypophysectomy the injection of ACT was begun.

The first injection period extended over 23 days; then the administration of hormone was discontinued for 10 days, after which it was resumed for a period of 6 days. During both courses the daily dose of hormone was 1.5 mgm, given intraperitoneally in 3 divided doses. The blood pressure rose rapidly during the first week of hormone injection, reaching an average level of 173 mm. Hg. which was slightly higher than the average hypertensive level preceding hypophysectomy. During the second and third weeks of hormone administration the blood pressure readings declined and finally levelled off,

Received for publication April 28, 1947.

[1] Aided by grants from the James Foundation and the Schering Research Fund (administered by the Medical Research Committee of the University of California) and from the John and Mary Markle Foundation.

the average blood pressure on the last day of injection being 140 mm. Hg. After an interval of 10 days in which no ACT was given the average blood pressure was 134 mm. Hg. During the second period of injection, which lasted 6 days, 3 of the animals showed no rise in blood pressure. The other 3

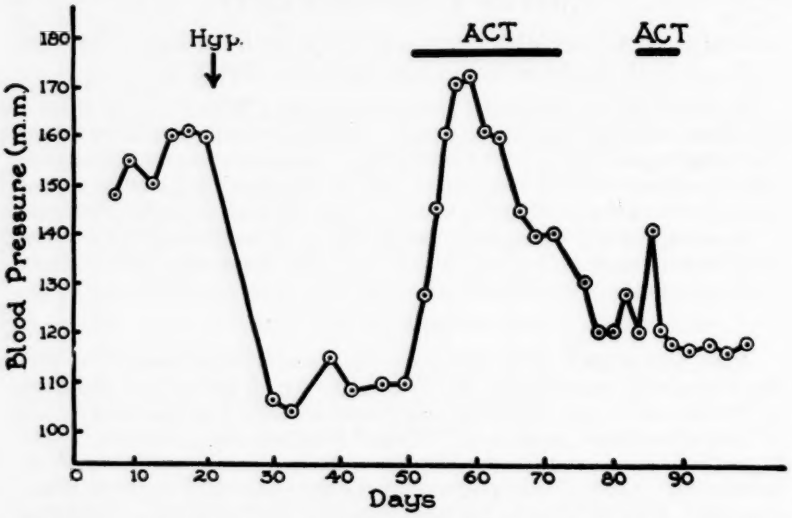


FIG. 1. Blood pressure response of rat B-9922 (see Table 1), which is representative of all the rats in the group.

TABLE 1. FAILURE TO MAINTAIN RENAL HYPERTENSION IN HYPOPHYSECTOMIZED RATS WITH CONTINUED ADMINISTRATION OF ACT

Animal number	Sex	Before hypophysectomy	After hypophysectomy	ACT for 23 days (dose = 1.5 mg./day)					No ACT for 10	ACT for 6 days dose = 1.5 mg./day
				Max. response		End of response	Final reading			
		B.P. (1)	B.P. (2)	B.P. (3)	day (4)	day (5)	B.P. (6)	B.P. drop (7)	(8)	B.P. response (daily reading) (9)
B-9900	F	158	130	168	4	11	130	−38		132, 128, 120, 123, 120
B-9928	M	159	128	161	6	13	135	−26		130, 160, 125, 130, 120
B-9922	F	160	110	172	9	18	138	−34		120, 128, 120, 142, 120
BH-9901	M	170	112	181	7	13	130	−51		128, 120, 130, 130, 130
BH-9927	F	155	128	172	9	18	135	−37		138, 138, 140, 135, 130
W-9905	M	157	127	182	13	21	139	−43		158, 135, 135

(1) Average of 6 readings taken during the 2 weeks before hypophysectomy. Hypertension established 8 weeks before hypophysectomy.
(2) Average of 4 readings taken during the second week after hypophysectomy.
(3) Highest blood pressure recorded.
(4) The number of days of injection before the peak of blood pressure was reached.
(5) The number of days of injection before blood pressure returned to pre-injection level.
(6) Blood pressure on the last day of injection.
(7) Difference between maximal response and final reading.
(8) No ACT given during the interval of 10 days.
(9) Blood pressure readings during a second course of ACT injections for 6 days.

exhibited a slight rise for one day only. The data are given in Table 1. Figure 1 shows graphically the blood pressure response of rat B-9922, listed in Table 1. The blood pressure curve of this animal is representative of the group.

DISCUSSION

Adrenal cortical hormone is necessary to maintain an elevated blood pressure in animals with partial occlusion of a renal artery (Goldblatt, 1937; Page, 1938; Page, Ogden and Anderson, 1946). The failure of ACT to maintain an elevated blood pressure in these animals is presumed to be due to a waning of its stimulating effect on the adrenal cortex. There have been numerous reported observations on the development of a refractory state of all of the anterior pituitary hormones except ACT. Thompson (1941) in an exhaustive review of the literature on the antihormones, referred to only one paper (Thompson and Cushing, 1934) in which is contained evidence of refractoriness to ACT. It is possible that other workers have obtained similar evidence of a refractory state to this hormone but have not interpreted their data on this basis. It remains to be shown that the serum of animals which have been injected with ACT over an extended period of time contains an adrenocorticotrophic antihormone, but this must wait until a more abundant supply of purified ACT is available.

SUMMARY

Hypertension was established in 7 adult rats by partial occlusion of the left renal artery. Hypophysectomy caused a fall in blood pressure from an average of 160 mm. Hg to 123 mm. Hg. The administration of a purified preparation of adrenocorticotrophic hormone restored the blood pressure to the pre-hypophysectomy level. However, despite continued injections of the hormone over a period of 23 days the blood pressure gradually fell to the pre-injection level. A second course of injections had virtually no effect on the blood pressure.

EVELYN ANDERSON,[2] ERNEST W. PAGE AND CHOH HAO LI

From the Institute of Experimental Biology and the Divisions of Medicine and of Obstetrics and Gynecology, University of California, Berkeley and San Francisco.

REFERENCES

ANDERSON, E., E. W. PAGE, C. H. LI AND E. OGDEN: *Am. J. Physiol.* 141: 393. 1944
GOLDBLATT, H.: *Ann. Int. Med.* 11: 69. 1937.
LI, C. H., H. M. EVANS AND M. E. SIMPSON: *J. Biol. Chem.* 149: 413. 1943.
PAGE, I. H.: *Am. J. Physiol.* 122: 352. 1938.
SMITH, P. E.: *J.A.M.A.* 88: 158. 1927.
THOMPSON, K. W.: *Physiol. Rev.* 21: 588. 1941.
THOMPSON, K. W., AND H. CUSHING: *Proc. Roy. Soc. London*, s.B. 115: 88. 1934.
WILLIAMS, T. R., T. R. HARRISON AND A. GROLLMAN: *J. Clin. Investigation* 18: 373. 1939.
WILSON, C., AND F. B. BYRON: *Lancet.* 1: 136. 1939.

[2] Fellow of the John Simon Guggenheim Memorial Foundation for 1946–47.

THE INFLUENCE OF THE THYROID ON ALANINE SYNTHESIS BY THE LIVER*

WE HAVE investigated the influence of thyroidectomy and of the administration of desiccated thyroid upon the capacity of rat liver to synthesize alanine from sodium pyruvate and ammonium carbonate. The animals used were young adults, both male and female, mostly about 5 or 6 months old, a few as old as a year. In the normal animal, we observed no correlation between either sex, or within the limits of our observations, age, upon the synthesis we were studying. The observations were made upon liver slices pre-

INFLUENCE OF THYROID ON SYNTHESIS OF ALANINE BY LIVER

	Normal	Thyroid-ectomized	Thyroid Fed		$\frac{\bar{X}}{\sigma}$
			Normal	Thyroid-ectomized	
	5.12 ± 0.364 (23)*	4.18 ± 0.680 (8)			Relation to normal 1.38
μg. NH_2N/mgm. dry wt. tissue/hr.			3.04 ± 0.242 (17)		4.76
				3.41 ± 0.630 (11)	2.20

* Figures in parentheses refer to the number of experiments.

pared as for the Warburg technique, the animals being killed by a blow. Tissues were sliced into 20 cc. of a bicarbonate buffered Krebs solution containing phosphate, and about 100 mgm. (dry weight) were transferred to 20 cc. of of the above solution, or in the test experiments, to the same solution containing 0.1 M sodium pyruvate and 0.04 M ammonium carbonate. The amount of alanine synthesis was inferred from the increase of amino acid nitrogen in the medium, as determined by the Van Slyke method. The experiments were of two hours' duration, in an overlying atmosphere of 5 per cent CO_2 and 95 per cent O_2, at the end of which time the tissues were filtered, washed and dried for weight. The alkalinized filtrate was boiled to get rid of ammonia, and was then analyzed for amino nitrogen by the method of Van Slyke. Since no amino nitrogen appeared when pyruvate and carbonate were incubated in the medium without tissue, it was assumed that the amino nitrogen appearing in the flasks containing tissues and medium represented the amount leached from the tissues. This was deducted from the quantity found in the

Received for publication April 28, 1947.

* Aided by a grant from the Committee on Endocrinology of the National Research Council, and the Charlton Research Fund, Tufts College Medical School.

test flasks, and the difference asumed to represent the synthesized amino nitrogen.

The results obtained are to be seen in the table. In the control group of normal animals, the alanine synthesized, expressed in terms of μg. of amino nitrogen per mgm. dry weight of tissue per hour, was 5.12 ± 0.364. This may be compared with the figure of 4.2 μg., which on recalculation, appears to have been synthesized by rat liver in the experiments of M. G. Kritsman (1944), using 0.1 M pyruvate and 0.02 M ammonium phosphate or acetate.

In a group of similar animals that had been thyroidectomized 6 to 10 weeks previously, the synthesis amounted to 4.18 ± 0.680. There was thus a tendency towards diminution of function, but the difference was not statistically significant.

In a group of normal animals that had been fed 500 mgm. of dessicated thyroid (U. S. P. Lilly) daily for ten days there was a marked reduction in alanine synthesis to a value 3.04 ± 0.242 μg. Since the value $\overline{X}/\sigma$ for this effect, where $\overline{X}$ is the difference in amino N synthesized, and σ is the square root of the sum of the squares of the standard deviations, is 4.76, the significance of the change is unmistakable. This finding is opposed to that of I. Zitovskaya, (1939), who found in normal dog's liver (according to our recalculation) a synthesis of 2μg. per mgm. dry weight per hour, which rose to 4.6 μg. in animals that have previously received 1 mgm. thyroxine daily for five days. When dessicated thyroid was fed to thyroidectomized animals in our experiments, the depression of function was somewhat less. This is explicable on the ground that the excess of circulating thyroid hormone, after the administration of the same amount of dessicated thyroid, would be less in the thyroidectomized animal.

We conclude that the hyperthyroid state depresses the amino acid synthesizing function of the liver.

Attilio Canzanelli, Ruth Guild and David Rapport
From the Physiology Department, Tufts College Medical School
Boston, Massachusetts

REFERENCES

Kritsman, M. G.: *Biochimia* 9: 382. 1944.
Zitovskaya, I.: *Bull. Biol. Med. Exptl. U.S.S.R.* 7: 114. 1939.